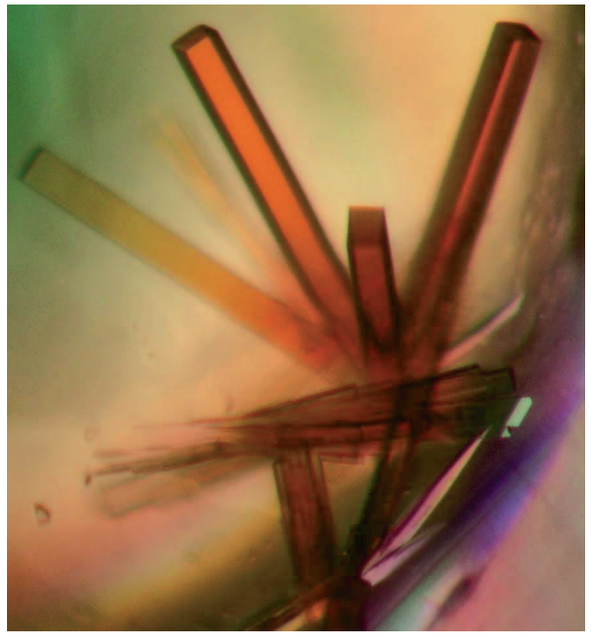
Thesis for doctoral degree (Ph.D.) 2010

Thesis for doctoral degree (Ph.D.)
2010

24S-HYDROXYCHOLESTEROL. STUDIES ON REGULATORY MECHANISMS BEHIND ITS FORMATION IN THE BRAIN AND ITS POTENTIAL USE AS A MARKER FOR NEURODEGENERATION



Marjan Shafaati

Karolinska Institutet

200 Years 1810 – 2010

24S-HYDROXYCHOLESTEROL Marjan Shafaati

Karolinska Institutet

200 Years 1810 – 2010

From the Department of Laboratory Medicine
Division of Clinical Chemistry
Karolinska Institutet
Stockholm
Sweden

24S-HYDROXYCHOLESTEROL. STUDIES ON REGULATORY MECHANISMS BEHIND ITS FORMATION IN THE BRAIN AND ITS POTENTIAL USE AS A MARKER FOR NEURODEGENERATION

Marjan Shafaati

Karolinska Institutet

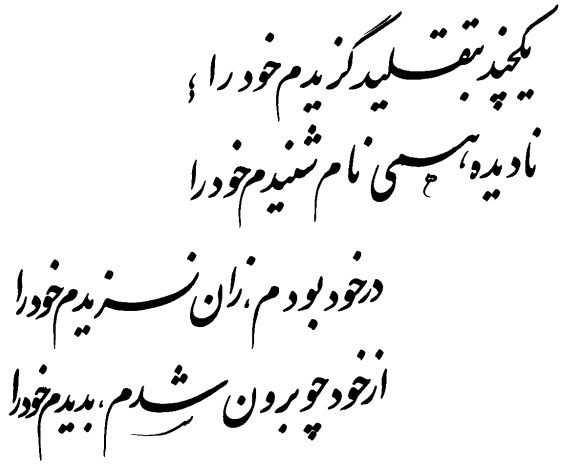
Stockholm 2010

Cover Illustration: Tetragonal crystals of CYP46A1.
Reference: White MA, Mast N, Bjorkhem I, Johnson EF, Stout CD, Pikuleva IA. Use of complementary cation and anion heavy-atom salt derivatives to solve the structure of cytochrome P450 46A1. Acta Crystallogr D Biol Crystallogr 2008 May;64(Pt 5):487-95.

Published by Karolinska Institutet.

ISBN 978-91-7409-978-2

Printed by
REPROPRINT AB
Stockholm 2010
www.reproprint.se
Gårdsvägen 4, 169 70 Solna



For a while, imitating others, I was proud of myself.
Innocent, I thought of myself as only a name.

When I was in my self I did not realize my true nature.
When I went beyond my self, then I realized my self.

Jalal al-Din Rumi,(Maulana) (1207-1273)

Dedicated to my parents

Thank you for giving up everything so I could have it all.

ABSTRACT

Cholesterol 24-hydroxylase (CYP46A1) belongs to the cytochrome P450 super family and is responsible for conversion of cholesterol to the oxysterol 24S-hydroxycholesterol (24S-OHC). This structural modification allows 24S-OHC to traverse the blood brain barrier and this pathway is the major one for elimination of cholesterol from the mammalian brain. CYP46A1 is almost exclusively located to neurons in the brain and retina. The promoter region of CYP46A1 shows classical hallmarks of a gene with putative housekeeping function. Oxidative stress was the only factor initially shown to cause significant increase in CYP46A1 reporter activity.
The aim of this thesis was to obtain a more profound knowledge about regulation of CYP46A1 and the regulatory importance of its product. In addition the possibility was investigated that the levels of 24S-OHC in cerebrospinal fluid may be used diagnostically.

In **paper I** we tested the possibility that epigenetic factors are important for CYP46A1 expression. We could demonstrate both *in vivo* and *in vitro* that the histone deacetylase inhibitors Valproate and Trichostatin A induce the expression of CYP46A1, essentially reprogramming non-neuronal cells to express CYP46A1 to the same level as that found in adult neurons.
In **paper II** we investigated the inhibitory effect of the antifungal drug Voriconazole on CYP46A1 in mice and we hypothesised that inhibition of CYP46A1 may have a role in the reported side effects in connection with Voriconazole (neurological and visual disturbances). A decrease in concentration of 24S-OHC in the mouse brain and a reduction in the lathosterol:cholesterol ratio, an index of brain cholesterol synthesis, was demonstrated .
In **paper III** we investigated the effect of omega-3 polyunsaturated fatty acids in Syrian Hamsters. Enrichment of diet with omega 3-fatty acids resulted in increased CYP46A1 mRNA levels in the brain.
In **paper IV** we investigated the consequences of an overexpression of CYP46A1. A transgenic mouse model overexpressesing human CYP46A1 was developed and characterized. Significant expression of the human CYP46A1 protein was found in brain, testis and eye and the brain in which contained more than 10-fold higher levels than the other organs. Circulating levels of 24S-OHC were increased by 4-6 fold and the fecal excretion of this steroid in free form was increased more than 20 fold. In the brain of the transgenic mice the total amount of CYP46A1 protein was increased 2-4 fold and had similar cellular distribution to the endogenous enzyme. The level of 24S-OHC in the brain was about double compared to the controls. It is known that 24S-OHC is an efficient ligand for the liver X receptor (LXR) *in vitro*. The overproduction of 24S-OHC did not significantly activate any LXR target genes in the brain, while in the liver some of these genes were affected but not in the direction expected in connection with LXR activation.
Based on *in vitro* studies a regulatory link between 24S-OHC and APOE has been suggested and addition of 24S-OHC stimulates secretion of APOE from cultured astrocytes. In **paper V** we investigated if there is a correlation between the concentration of APOE and 24S-OHC in cerebrospinal fluid in patients. A significant

correlation was found in patients with Alzheimer's disease (AD) and mild cognitive impairment (MCI), but not in the control group.
In **paper VI** we investigated the possibility that the level of 24S-OHC in cerebrospinal fluid may be an early biomarker for AD. We could show that 24S-OHC is as sensitive as the standard diagnostic biomarkers for AD (tau-protein, phospho-tau, and beta amyloid). Interestingly 24S-OHC appeared to be a more sensitive marker for MCI than the standard biomarkers.

According to current concepts the levels of cholesterol in critical neuronal membranes is of importance for the balance the between α- and β- secretase pathway. An increased consumption of this cholesterol by increased activity of CYP46A1 would be expected to favour the α-secretase pathway and reduce amyloid formation. Under *in vitro* conditions 24S-OHC seems to have a direct inhibitory effect on amyloid formation. The mouse model with overexpressed CYP46A1 presented in *paper IV* will be ideal to test the hypothesis that an upregulation of CYP46A1 is neuroprotective and reduces amyloid formation. As shown in *paper I* histone deacetylase inhibitors are potential drugs to reach this goal. High intake of omega-3 fatty acids seems to have some neuroprotective effects in humans and the results shown in *paper III* is consistent with the possibility that part of this may be mediated by an effect on CYP46A1. Inhibition of CYP46A1 would be expected to have a negative effect on the function of the central nervous system and possibly also retina. The results of *paper II* demonstrated that the drug Voriconazole, with known negative side effects on CNS and visual function, inhibits the flux in the mevalonate pathway in the brain. In part this may be the consequence of inhibition of CYP46A1. Results are presented to indicate that the levels of 24S-OHC in cerebrospinal fluid may be used as diagnostic tool in connection with neurodegeneration (*paper V and VI)*.

LIST OF PUBLICATIONS

I. *Transcriptional regulation of cholesterol 24-hydroxylase by histone deacetylase inhibitors.*
Shafaati M, O'Driscoll R, Björkhem I, Meaney S.
Biochem Biophys Res Commun. 2009 Jan 23;378(4):689-94.

II. *The antifungal drug Voriconazole is an efficient inhibitor of brain cholesterol 24S-hydroxylase in vitro and in vivo.*
Shafaati M, Mast N, Beck O, Nayef R, Heo GY, Björkhem-Bergman L, Lütjohann D, Björkhem I, Pikuleva IA.
J Lipid Res. 2010 Feb;51(2):318-23.

III. *Marked variability in hepatic expression of cytochromes CYP7A1 and CYP27A1 as compared to cerebral CYP46A1. Lessons from a dietary study with omega 3 fatty acids in hamsters.*
Mast N, **Shafaati M**, Zaman W, Zheng W, Prusak D, Wood T, Ansari GA, Lövgren-Sandblom A, Olin M, Björkhem I, Pikuleva I.
Biochim Biophys Acta. 2010 Jun;1801(6):674-81.

IV. *Metabolic consequences of an overexpression of the cholesterol 24S-hydroxylase (CYP46A1) in the mouse*
Shafaati M*, Maria Olin*, Ann Båvner, Hanna Pettersson, Björn Rozell, Steve Meaney, Paolo Parini, Ingemar Björkhem
Manuscript

V. *Levels of ApoE in cerebrospinal fluid are correlated with Tau and 24S-hydroxycholesterol in patients with cognitive disorders.*
Shafaati M, Solomon A, Kivipelto M, Björkhem I, Leoni V.
Neurosci Lett. 2007 Sep 25;425(2):78-82.

VI. *Are the CSF levels of 24S-hydroxycholesterol a sensitive biomarker for mild cognitive impairment?*
Leoni V, **Shafaati M**, Salomon A, Kivipelto M, Björkhem I, Wahlund LO.
Neurosci Lett. 2006 Apr 10-17;397(1-2):83-7.

*Authors contributed equally to the work.

CONTENTS

LIST OF ABBREVIATIONS

Aβ	Amyloid beta
ABCA1	ATP-binding cassette, sub-family A , member 1
ABCG	ATP-binding cassette gene
ACAT	Acyl CoA Cholesterol transferase
AD	Alzheimer's Disease
AMPK	AMP-activated protein kinase
AMP	Adenosinemonophosphate
ALA	α-linoleic acid
APOA	Apolipoprotein A
APOB	Apolipoprotein B
APOC	Apolipoprotein C
APOD	Apolipoprotein D
APOE	Apolipoprotein E
APOJ	Apolipoprotein J
APP	Amyloid precursor protein
ATP	Adenosine triphosphate
BBB	Blood brain barrier
CA	Cholic Acid
CDCA	Chenodeoxycholic acid
cDNA	Complementary DNA
DHA	Docosahexaenoic acid
CNS	Central nervous system
CSF	Cerebrospinal fluid
CYP46A1	Cholesterol 24-hydroxylase
CYP27A1	Sterol 27-hydroxylase
EEG	Electroencephalography
ELISA	Enzyme-linked immunosorbent assay
EPA	Eicosapentanoic acid
GC	Guanin-Cytosine
GC-MS	Gas chromatography-mass spectrometry
ER	Endoplasmic reticulum
HAT	Histone acetyltransferase
HDACi	Histone deacetylase inhibitor
HDL	High-density lipoprotein
HMG-CoA	3-hydroxy-3-methyl-glutaryl-CoA
HMGCR	3-hydroxy-3-methyl-glutaryl-CoA reductase
INSIG	Insulin induced gene
LDL	Low-density lipoprotein
LDLR	Low-density lipoprotein receptor
LOAD	Late onset sporadic AD
LXR	Liver X receptor
MMSE	Mini-mental state examination
MRI	Magnetic resonance imaging
MCI	Mild cognitive impairment

NADPH	Nicotinamide adenine dinucleotide phosphate-oxidase
NMDA	N-metyl-D-aspartat
NPC	Niemann-Pick type C
PUFA	Polyunsaturated fatty acids
SCAP	SREBP cleavage activating protein
SP	Specificity protein
SPECT	Single photon emission computed tomography
SREBP	Sterol regulatory element binding protein
SRE	Sterol regulatory element
SSH	Sonic hedghog
TSA	Trichostatin A
VLDL	Very low density protein
24S-OHC	24S-hydroxycholesterol
27-OHC	27-hydroxycholesterol

INTRODUCTION

Milestones in the history of cholesterol

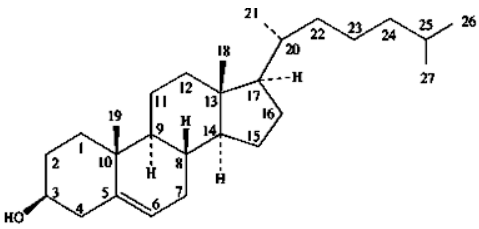


Figure 1. Chemical structure of the cholesterol molecule

The French chemist Michel Eugène Chevreul is accredited with the initial isolation of cholesterol (Fig. 1) in 1815, but it was not until 1888 that the empirical formula $C_{22}H_{46}O$ was established by Reinitzer. The correct chemical structure of the molecule was discovered by Wieland and Dane in 1932. However, already in 1834, Couerbe recognized the importance of cholesterol in the nervous system (1, 2). Cholesterol is the molecule most decorated by Nobel prizes, the most recent of which was awarded to Michael S. Brown and Joseph I. Goldstein in 1985 for their discoveries concerning the regulation of cholesterol metabolism. We have come a long way in understanding the biology of this molecule and one can only be astonished by the endless discoveries that have been revealed since the discovery of cholesterol. Considerably less is known about cholesterol turnover in the brain than in other organs.

Biological roles of Cholesterol

Cholesterol is a multi-functional molecule. Among its many functions it acts as a precursor for steroid hormones and bile acids. It is also a critical component of cellular membranes where it affects membrane fluidity. Cholesterol homeostasis must be tightly controlled throughout the organism to avoid excess deposition or decreased synthesis and this is particularly the case in the brain. Cholesterol has a pivotal function in the central nervous system (CNS) where it serves as a critical component of the myelin sheath and is involved in synapse formation. (2).

The myelin is important for action potential velocity. The evolutionary adaption of the cholesterol–rich plasma membrane to form compact myelin made it possible to "wire" the complex brain to a very condensed structure with relatively small axon diameter.
Cholesterol is also important for the normal development of the brain and is critical for the correct targeting of the morphogenic factor Sonic hedgehog which drives the expansion of the largest neuronal population in the brain. Inhibition of Sonic hedgehog leads to abnormal brain development (3).

Synthesis of cholesterol

A normal healthy adult synthesizes approximately 1g of cholesterol every day and consumes about 0,4-0,5 g/day (4). A constant level of cholesterol in human plasma of about 1,5-2,0 g/L (4-5mM) is maintained by control of its synthesis as well as its uptake and metabolism. The level of cholesterol is influenced to a small degree by the dietary intake of cholesterol. Newly synthesized cholesterol is used in the formation of membranes and in the synthesis of steroid hormones and bile acids (4).

Biosynthesis of cholesterol requires large amounts of energy and involves numerous enzymes in the cytosol and the endoplasmic reticulum (ER). Cholesterol is made from Acetyl-CoA in four stages: 1) condensation of three acetate units to form a six-carbon intermediate, mevalonate; 2) conversion of mevalonate to activated isoprene units; 3) polymerisation of five-carbon isoprene units to form squalene; and 4) cyclization of squalene to form the four rings of the steroid nucleus, followed by a series of oxidations, reductions and removal of methyl groups which lead to cholesterol formation. The rate-limiting step in the pathway to cholesterol is the conversion of 3β-hydroxy-3-methylglutaryl-coenzyme A (HMG-CoA) to mevalonate, a reaction catalyzed by the microsomal HMG-CoA reductase (HMGCR) that can be inhibited by statins (5). Sterol regulatory element binding proteins (SREBP) are a family of transcription factors that regulate cellular cholesterol and fatty acid homeostasis by responding to the cellular levels of free cholesterol. SREBPs bind as dimers to Sterol Regulatory Elements (SREs) in the promoter region of a large number of genes resulting in increased transcription. There are three SREBP isoforms: SREBP-1a, 1c and 2. The first two SREBPs are thought mainly to regulate the genes involved in fatty acid synthesis whereas SREBP-2 mainly regulates the genes involved in cholesterol biosynthesis. SERBP-1a is believed to activate genes in both processes. SREBP-2 activates its own transcription in combination with another transcription factor, nuclear factor Y. In the regulation of cholesterol synthesis SREBP interacts with two other proteins, insulin induced protein (INSIG) and SREBP cleavage activating protein (SCAP).

SCAP is a membrane bound protein that has a sterol sensing domain similar to that in HMGCR. In the presence of high concentrations of cholesterol SREBP remains bound to the ER through an INSIG-1: SCAP complex. Decreasing cholesterol levels lead to a conformational change in SCAP which results in release of INSIG-1. This is the consequence of enzymatic cleavage of SREBP in the SCAP: SREBP-2 complex in a two-step process by two membrane bound proteases, site 1 protease and site 2 protease. The released amino-terminal active form of SREBP-2 then moves to the nucleus and binds to promoters containing SREs (6-9).

HMGCR can also be regulated by phosphorylation and dephosphorylation. The enzyme is most active in its unmodified form and phosphorylation of the enzyme decreases its activity. HMGCR is phosphorylated by adenosine monophosphate activated protein kinase, AMPK. The activity of HMGCR is additionally controlled by the cyclic adenosine monophosphate signalling pathway. Increase in cAMP leads to activation of cAMP-dependent protein kinase. Since the intracellular level of cAMP is regulated by hormonal stimuli, regulation of cholesterol biosynthesis is hormonally controlled to some extent (10).

The ability of insulin to stimulate, and glucagon to inhibit HMGCR activity is consistent with the effects of these hormones on other metabolic pathways.

Long-term control of HMGCR activity is exerted primarily through control over the synthesis and degradation of the enzyme. When levels of cholesterol are high, the level of expression of the HMGCR gene is reduced. Conversely, reduced levels of cholesterol activate expression of the gene. Insulin also regulates cholesterol metabolism by increasing the level of HMGCR synthesis. HMGCR is localized to the ER and like SCAP contains a sterol-sensing domain. High sterols levels cause the INSIG proteins to bind to HMGCR sterol-sensing domain; an ubiquitin ligase (gp78) is recruited to the ER and ubiquitinates HMGCR and targets it for the proteasome, a multiprotein complex dedicated to protein degradation (11). The primary sterol regulating HMGCR degradation appears to be cholesterol itself. As the levels of free cholesterol increase in cells, the rate of HMGCR degradation increases.

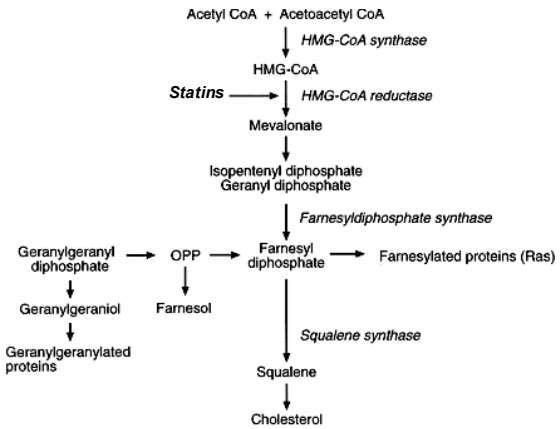


Figure 2. Overview of cholesterol biosynthesis (12). Note that each arrow is actually several steps and the branch pathways are omitted for clarity.

Cholesterol absorption, transport and metabolism to bile acids

The cholesterol in the diet is absorbed from the intestine and ultimately delivered to the liver by chylomicrons. In the liver cholesterol can be converted to bile acid or secreted to extrahepatic tissues as very low density lipoproteins (VLDL). VLDL are remodeled by the action of lipoprotein lipase that removes core triacylglycerol molecules and Apolipoprotein A (APOA1) and Apolipoprotein C (APOCI and ACPOCII) from VLDL to high density lipoprotein (HDL). Ultimately this process results in the formation of a low density lipoprotein (LDL) particle which can supply peripheral tissues with cholesterol. The cellular LDL intake is tightly regulated via the LDL receptor (LDLR) and Apolipoprotein B (APOB). The influx of cholesterol inhibits HMGCR and cholesterol synthesis, and upregulates cholesterol esterification by acyl-CoA:cholesterol Acyl transferase (ACAT1). Reverse cholesterol transport, by which cells from different organs eliminate excess cholesterol is mediated by HDL. The HDL particles contain APOA1 which can extract cholesterol directly from the plasma membrane. This transfer is facilitated by members of the ATP binding cassette (ABC)-transporter family.

About 1g of cholesterol is eliminated from the body every day. Approximately half of this is excreted into the feces after being converted to bile acids. The bile acids formed

play an important role in solubilisation and absorption of fats, cholesterol, lipophilic vitamins and certain drugs. There are two major pathways of bile acid synthesis: the neutral pathway is initiated by the rate-limiting enzyme cholesterol 7α-hydroxylase, (CYP7A1) whereas the alternative or acidic pathway is initiated by the enzyme sterol 27-hydroxylase (CYP27A1). Under normal conditions the neutral pathway dominates in healthy adult humans (13). In humans the bile acid pool consists of the primary bile acids cholic acid (CA) and chenodeoxycholic acid (CDCA) and also the secondary bile acids deoxycholic acid (DCA) and lithocholic acid. CA is considered to be a product of the classic pathway whereas CDCA is the major end product of the alternative pathway.

In addition to this the liver can eliminate cholesterol through excretion of free cholesterol in bile via the transporters ABCG5 and ABCG8. Disruption in the latter transport system leads to severe sitosterolemia and patients with this condition have an accumulation of plant sterols and an increased risk of atherosclerosis. Activation of ABCG5/G8 transcription by the nuclear receptor liver X receptor (LXR) results in increased cholesterol excretion (14).

Cholesterol in CNS

Cholesterol has an important role in connection with brain development and function. Cerebral cholesterol represents 2-3% of the wet weight of the brain, corresponding to about 25% of the total amount of cholesterol present in humans. The cholesterol content of the brain is independent of the dietary uptake and hepatic synthesis and there is solid evidence that almost all of it is produced *in situ*. Brain cholesterol is isolated from other pools of cholesterol by the blood-brain barrier (BBB)(2,15,16). The synthesis of cholesterol during the development of the central nervous system is relatively high but declines to very low levels in the adult stage (16). This is possible due to the efficient recycling of the brain cholesterol. As a consequence of this, and the fact that myelin is almost excluded from the general metabolism, brain cholesterol has an extremely long half-life(2). Despite this efficient mechanism of reutilization, there are specific pathways for cholesterol elimination from the brain. It has been estimated that 1-2 mg cholesterol may be eliminated from the brain via the cerebrospinal fluid (CSF) in apolipoprotein E (APOE) containing lipoproteins each day as part of normal CSF turnover (2). About 6-7 mg is eliminated by the 24S-hydroxylase (CYP46A1) pathway (*cf*. below)

In the CNS cholesterol is present in 2 major pools; myelin cholesterol that is a product of oligodendroglia and cholesterol present in plasma membranes of astrocytes and neurons (16).

The astrocytes are believed to be responsible for most of the synthesis of cholesterol required for the neurons. They secrete cholesterol in APOE containing lipoproteins. ABCA1 is a monomeric transporter that resides in the plasma membrane of tissues including liver, intestine, placenta, adipose, spleen and brain. It transports phospholipids and cholesterol while (ABC binding cassette gene 1) ABCG1 has been implicated in intracellular trafficking of sterols in macrophages (14). Astrocytes also synthesise and secrete APOE. This process has been shown to be stimulated by 24S-OHC. In the present work we study the possibility that there is a relation between 24S-OHC and APOE in CSF *(paper IV*).

APOE-bound cholesterol enters the neurons via the LDLR. Once in the cell cholesterol passes the post-lysosome, late endosome system via the Niemann-Pick type C (NPC)

proteins type 1 (NPC1) and 2 (NPC2). NPC1 is a large membrane that localizes in the endosome and NPC2 is a small luminal protein that binds cholesterol with high affinity. Cholesterol can then be transported to the plasma membrane via vesicular transport or delivered to organelles (17).
Cholesterol is converted into 24S-OHC via CYP46A1 in the neurons. In contrast to cholesterol this monooxygenated molecule is able to traverse the BBB (2, 19). It has been shown that elimination of cholesterol from the brain of rats (20) and mice (21) corresponds to about 2/3 of the cholesterol synthesis in this organ.

24S-OHC and other oxysterols are ligands of the LXR, a class of nuclear hormone receptors (22), which translocate to the nucleus and may induce expression of both APOE and ABCA1 genes in astrocytes (18). LXR subtypes are important components of a complex regulatory system that senses cholesterol levels and modifies gene expression accordingly (22). The isoforms LXRα and β have been identified and are known to be activated by oxysterols *in vitro*. LXRα is restricted to certain tissues and LXR β is more generally expressed. LXR target genes include ABCA1 and ABCG1, and SREBP1.
In the CNS both of the isoforms are expressed and believed to be involved in the brain cholesterol metabolism. LXR β levels are 2-5 folds higher in the brain than in liver.
Levels of LXRα in cultured neurons and glia are 2% and 17% compared to the liver, and that for LXR β are 110% and 380%, respectively (23).
LXR double knock-out mice show a variety of CNS defects upon aging including lipid accumulation, astrocyte proliferation, and disorganization of the myelin sheaths. Both cholesterol and APOE are crucial for control of synaptic function. A normal cycle of cholesterol biosynthesis, transport, and turnover is required for normal synapse formation, stabilization, function and plasticity.
Neuronal cells seem to be dependent upon a flux of cholesterol from astrocytes (24). Based on in vitro experiments the possibility has been suggested that this flux may be regulated by 24S-hydroxycholesterol (24S-OHC) (Fig 3).
If the relation between Apo E and 24S-OHC suggested by the *in vitro* experiments by Pfrieger (24) and Abildayeva et al. (18) is of importance under *in vivo* conditions a relation between these compounds may be expected in CSF. This possibility was studied in *paper V*.

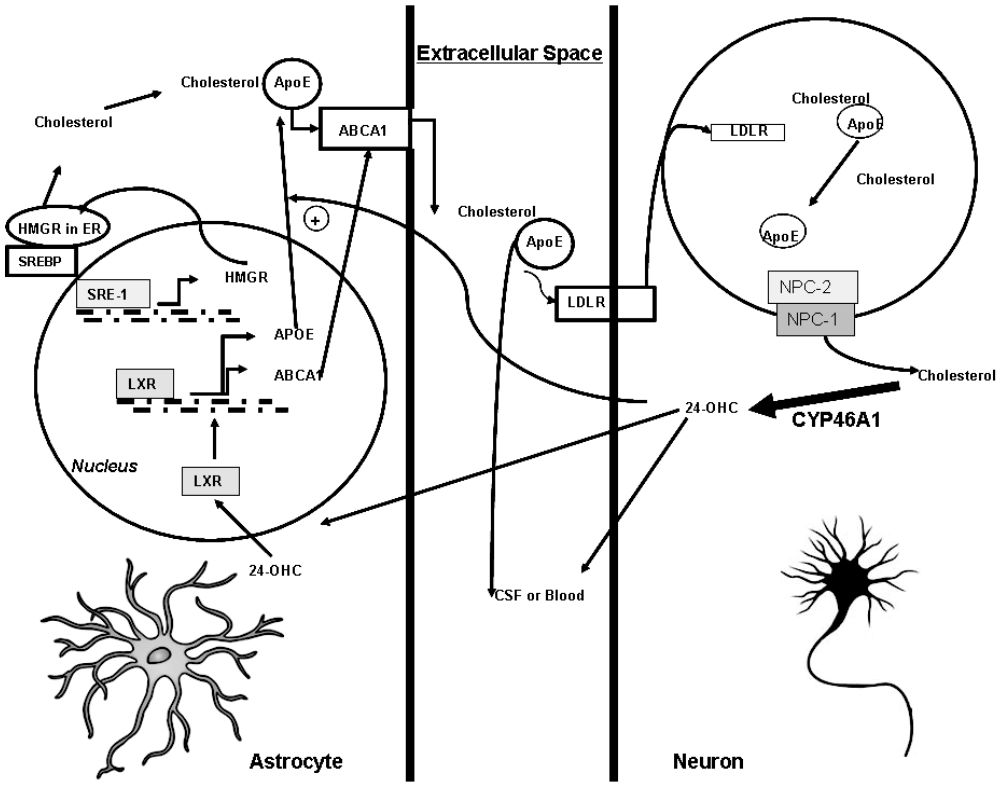


Figure 3. Suggested interaction between astrocytes and neuronal cells in cholesterol homeostasis modified according to Pfrieger (24) and Abildayeva et al (18). The neuronal cells are dependent on astrocytes for delivery of cholesterol. Excess cholesterol may be eliminated through the 24S-hydroxycholesterol mechanism. 24S-hydroxycholesterol is a potent activator of the nuclear receptor LXR. The latter activation may lead to increased activation of the cholesterol transporter ABCA1.

Lipoproteins

In the aqueous environment of the blood, neutral lipids circulate packaged as lipoproteins. Lipoproteins are composed of phospholipids and free cholesterol shell surrounding a triglyceride (TG) and cholesteryl-ester (CE) core. Lipoproteins are stabilized by surface lipoprotein and are ligands for lipoprotein receptors. Plasma lipoproteins are generally separated by size and density in four major classes that vary in their core TG/CE content and apoprotein composition: Chylomicrons, VLDL, LDL and HDL (25).

In the CNS including CSF the lipoproteins have a density similar to HDL, with a core of lipid and esterified CE. CSF lipoproteins are categorized by apolipoprotein into APOE, A-I, A-IV, APOD, APOH and APOJ (26, 27). APOE is the dominant apolipoprotein in the brain.

Apolipoprotein E

APOE was recognised in 1973 as part of triglyceride-rich lipoprotein complexes and it was shown to be important for cholesterol transport. APOE is expressed in several organs, with the highest expression in the liver followed by the brain (28). Astrocytes and to some extent microglia, are the major cell types expressing APOE (29-31). In humans the APOE genes show polymorphism with three different alleles (ε2, ε3 ,ε4) on chromosome 19, which give rise to six different phenotypes (E2/2, E2/3, E2/4, E3/3, E3/4 and E4/4).

The APOE isoforms differ only by a single amino acid substitution of Cys to Arg at position 112 and 158 (32) leading to different biological properties (33). APOE 3 is the most common isoform (77-78%) in the general population while APOE2 is found in 7-8% and APOE4 in 14-16% of individuals (34, 35). After findings of immunoreactivity of APOE in the amyloid plaques (36) the APOE4 allele was discovered to be the most important genetic risk factor for sporadic Alzheimer's disease (AD) (32, 37). In contrast, the APOE2 allele has been associated with a lower risk for AD (38). Amyloid beta (Aβ) is formed by proteolytic cleavage of the amyloid precursor protein (APP) and plays an important role in the AD pathology. There is evidence suggesting that APOE4 is somehow involved in the Aβ formation. As mentioned above, APOE is present in the neuritic plaques and it has been reported that Aβ levels are elevated in brains of AD patients carrying APOE4 allele. It is not yet known if APOE4 has an active role in aggregation and/or deposition of Aβ. APOE4, in a lipid- free form has a greater avidity to Aβ than APOE3 (34, 39, 40). Post mortem studies have shown increased Aβ deposition in APOE4 carriers both in sporadic and genetic AD cases (39, 41). Hyper-phosphorylation of microtubule-associated protein known as tau protein leads to insoluble aggregation and form neurofibrillary tangles in the neuronal cells. This is common in connection with neurodegenerative diseases, in particular AD. Different APOE isoforms may influence formation of tau. *In vitro* studies have shown that APOE3 forms a stable complex with tau. The interaction between APOE3 and tau was prevented by phosphorylation of tau, suggesting that APOE3 preferentially binds to nonphosphorylated tau (42). However there is no evidence demonstrating localization of APOE to the neuronal cytosol, where the majority of tau exists under normal conditions (29, 43). In the peripheral nervous system and CNS the levels of APOE increases following neuronal injuries. It has been suggested that this increase may be required for repair of the nervous system by redistribution of lipids and cholesterol for membrane repair and synaptic plasticity. Most studies have shown that APOE3 augment neurite outgrowth to a greater extent than APOE4 (29, 44). The effect of APOE2 has not yet been fully examined in connection to neurite sprouting.

Other lipoproteins in the brain

In addition to APOE and APOAI, APOJ and APOD are important lipoproteins in the brain. Interestingly the apolipoproteins appear to have overlapping functions. A knockout of APOE results in a marked compensatory increase in levels of APOD (45).

APOJ is a 70kDa protein associated with HDL in human plasma. This multifunctional ubiquitous protein has been purified from several tissues (46-48). The adult brain is a major site of APOJ mRNA synthesis in several mammalian species (46, 47, 49, 50). This lipoprotein has been associated with many different injuries and chronic inflammation of the brain including Multiple Sclerosis (51), Huntington disease (52) and

AD (53). Experimental brain lesions induce a marked increase of APOJ in neurons. Aging appears to up-regulate the expression in neurons (54). In AD increased levels of hippocampal and cortical APOJ mRNA and protein have been reported (55). APOJ has been suggested to have several neuroprotective properties: 1) direct neuroprotection, through the triggering of signalling cascades that affect neurodegenerative pathways and apoptotic processes; 2) indirect neuroprotection, through its interaction with Aβ peptides and 3) neurotrophic functions, promoting general recovery from neuronal injury via lipid transport or membrane recycling (46, 56).

APOD is a secreted glycoprotein assigned with many putative functions including lipid transport. Human APOD was first identified in plasma HDL (57). In humans, its main sites of expression are the brain and testes. In the CNS, it is mainly expressed in glial cells (both astrocytes and oligodendrocytes) and their precursors, (58) but in pathological situations it can also be expressed in neurons. The protein itself is small (18 kD), soluble and has no homology to other apolipoproteins (59). Elevated levels of APOD have been described in association with various neurological disorders, including AD, Parkinson's disease and stroke. (59)
Since APOD is a lipid carrier, it is particularly interesting to observe its upregulation in disorders of the myelin. One of the most striking increases is observed in regenerating and remyelinating sciatic nerve, in the rat. After 3 weeks post-crush injury, APOD increases 500-fold at the site of the lesion, and remains elevated while regeneration takes course. (60)

Side-chain oxidized oxysterols
Oxysterols were first recognized by Lifschütz 100 years after the actual discovery of cholesterol. They are defined as mono-oxygenated derivatives of cholesterol. Several cytochrome P450 enzymes are involved in the formation of particular hydroxycholesterols. CYP46A1 is a microsomal enzyme present in neurons and produces 24S-OHC which is also called *cerebrosterol*, because of its abundance in brain. The mitochondrial enzyme CYP27A1 is responsible for the formation of 27-hydroxycholesterol (27-OHC) which is an intermediate in bile acid synthesis and is the major oxysterol in the human circulation.
In contrast to cholesterol the side chain oxidized oxysterols are able to traverse the BBB. The cross-over is dependent on the presence of a hydroxyl group in the side-chain of cholesterol leading to re-arrangement of membrane phospholipids in a way that is more favourable from an energy point of view to expel the oxysterol. Due to this, the flux of a side-chain oxidized oxysterol across a biomembrane may be three orders of magnitude faster than that of cholesterol. Most oxysterols have a short half-life because of the rapid conversion into bile acids and final elimination in the liver. The net efflux of 24S-OHC from the brain has been determined to be about 6-7mg per 24 hours (61).There is a corresponding uptake of the latter oxysterol from the liver. This demonstrates that the brain is the major source of the 24S-OHC present in the circulation.
Oxysterols are able to regulate the expression of many genes involved in lipid biosynthesis, at least under *in vitro* conditions (2,7,62,63).
They are also precursors to bile acids. Synthesis and secretion of some oxysterols by some tissues can be regarded as an alternative to reverse cholesterol transport by which excess sterol is transported to the liver for further catabolism (63).

27-OHC is formed in most extrahepatic organs but produced in particularly high quantities by macrophages. At high concentrations of the enzyme CYP27A1 this sterol may be further oxidized into a steroid acid (3β-hydroxy-5-cholestanoic acid) that can be eliminated from the cell even more efficiently than 27-OHC. Our laboratory has demonstrated an influx of 27-OHC into the human brain of about 5mg per 24h (64). This flux reflects the integrity and function of the BBB. Damage of the BBB thus shows higher levels of 27-OHC in the CSF (65, 66). High levels of 27-OHC in CSF may also reflect neuronal damage, because of the fact that the metabolizing enzyme CYP7B1 is connected to neuronal cells.

Sterol 27-hydroxylase; *CYP27A1*

CYP27A1 seems to have a rather stable activity and appears to be mainly regulated by substrate availability i.e. cholesterol concentration. Investigations in humans have shown no significant difference in transcription levels in the liver at different fluxes of bile acid (67). However, the addition of cholesterol to human extrahepatic cells results in a significant increase in CYP27A1 activity. This means that the stimulation is mainly due to increased substrate availability. The protein CYP27A1 consists of 531 amino acids and has a molecular weight of 60 kDa. Two other members of the CYP27 family have been identified: CYP27B1 is the important enzyme in connection to bioactivation of vitamin D; CYP27C1 has 43% sequence homology to CYP27A1 but the role of this form is still under investigation (68). CYP27A1 is an important enzyme involved in the acidic pathway for bile acid biosynthesis. As CYP27A1 is present in most cell types, extrahepatic 27-hydroxylation represents a quantitatively important extrahepatic pathway for bile acid synthesis.

Cholesterol 24-hydroxylase; *CYP46A1*

CYP46A1 is expressed in a subset of metabolically active neurons, such as pyramidal neurons of the hippocampus, hippocampal and cerebellar neurons, cortical neurons and the purkinje cells of the cerebellum (69). These cells display a very high rate of cholesterol turnover. The enzyme requires nicotinamide adenine dinucleotide phosphate-oxidase (NADPH) and oxygen for the conversion of cholesterol to 24S-OHC in brain microsomes (70). The enzyme consists of ~500 amino acids and has a broad substrate specificity. There are a number of endogenous and exogenous substrates for this enzyme (71, 72). Cloning of the mouse and human complementary DNA (cDNA) revealed unique qualities of the P450s (70). The two different 24-hydroxylases share less than 35% sequence identity with other members of the P450 super family. In general human and mouse P450 orthologs are ~70% identical in sequence while the CYP46A1 from these two species are 95% identical (73). Polyproline motifs like ProAlaProProProProPro-Cys or similar sequences are found at the extreme C termini of the mouse and human proteins. This feature is also present in the chimpanzee, rhesus, cow, dog, horse, and rat cholesterol 24-hydroxylases but is absent in the frog, chicken, zebra fish, and platypus enzymes (74). Other P450s lack such features. These motifs are usually binding sites for interacting proteins that are of importance for the function of this enzyme (69). No such interacting partners have yet been identified. The structure of the enzyme is organized into 12 α-helices and four β-pleated sheets. The heme prosthetic group is positioned between two α-helices and is liganded to cysteine 437 of the protein. An active site with a small volume (~300 Å3) is present in which a high-affinity substrate (cholesterol 3- sulfate) is bound via a combination of hydrogen bonding and hydrophobic packing with amino acids from

multiple α-helices (69, 75). Based on binding studies with the pure enzyme Mast et al. (72) defined a number of inhibitors. In *paper II* we study the possibility that one of these also inhibitors, Voriconazole, has an inhibitory effect on CYP46A1 *in vivo*.

Investigations on the promoter region of CYP46A1 showed classical features of a gene with a housekeeping function (73). The gene has a TATA-box or CAAT box free promoter, multiple transcription sites and a high guanine-cytosine (GC) content of the proximal part of the promoter, features often found in genes with house keeping function (70, 73). CYP46A1 appears to be insensitive towards a number of regulatory factors and experiments with a variety of promoter constructs and hormonal factors did not result in significant reporter activity.,oxidative stress, however, caused a significant increase in gene reporter activity (73). The GC rich region of the CYP46A1 promoter contains a number of putative specificity protein (Sp) binding sites (73) which may be of importance in connection with oxidative stress (76, 77). In this connection a recent work by Milagre *et al.* is of interest (78), demonstrating that Sp3 and Sp4 binding is required for high levels of CYP46 promoter activity. Little is thus known about the transcriptional modulation of this gene by endogenous or exogenous signals. In the present work we have studied the possibility that epigenetic mechanisms may be important for the regulation of the enzyme. Unlike most P450s that are expressed in liver, kidney, and lung, CYP46A1 expression is almost exclusively confined to the brain in the mouse and human (69). In the mouse, small amounts of Cyp46a1 mRNA may be detected in the testis and liver, but the mRNA does not appear to be translated into protein in the testis, and levels of the mRNA and protein are estimated to be approximately 100-fold lower in the liver than in the brain (69). The mouse embryo expresses the gene at day 11, with a gradual increase in both mRNA and protein expression. In humans the steady state expression level is reached after approximately 1 year and is maintained during adulthood (69, 73). The expression of the enzyme is thus regulated by unknown mechanisms during the first period of life. It has been shown in mice that CYP46A1 mRNA levels increase in parallel with the levels of 24S-OHC and a decrease of cholesterol synthesis during the first two weeks of life (73).
Recent reports on CYP46A1 knock out mice indicate that synthesis and turnover of cerebral cholesterol via 24-hydroxylation may play an important role for memory and learning ability. Kotti et al. reported that mice lacking CYP46A1 have reduced N-methyl-D-aspartate receptor mediated long-term potentiation in hippocampus and memory defects (80). This could be due to the feedback inhibition of HMGCR by decreased cholesterol concentration, leading to reduced flux in the mevalonate pathway.

Epigenetic regulation
Our failure to demonstrate a transcriptional regulation of CYP46A1 by the factors involved in cholesterol homeostasis in extracerebral tissues, led us to consider the possibility of an epigenetic type of regulation *(Paper I)*. This type of regulation involves acetylations, methylations, de-acetylations and de-methylations of histones.
Histone deacetylase inhibitors (HDACi) are a relatively new class of pharmacological agents capable of regulating the rate of gene transcription by hyperacetylation of histones. There are 5 different classes of inhibitors: 1) hydroxamic acid; 2) short-chain fatty acids; 3) synthetic benzamide derivatives; 4) cyclic tetropeptides and 5) miscellaneous compounds (81). Valproate and Trichostatin A (TSA) are two often used

HDACi. Recent interest in HDACi has grown due to their potential use in anti-cancer treatments and the recognition that histone modifications are of importance for memory. HDACi's can induce recovery of tumour suppressor genes, growth arrest, differentiation and apoptosis (82).

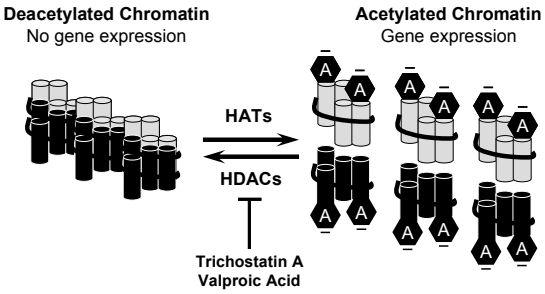


Figure 4. The effect of histone deacetylase inhibitors Valproate and Trichostatin A on gene expression. The action of HDACi's results in hyperacetylation of histone tails. HDACi's inhibit histone deacetylases allowing activation of histone acetyltransferases. This acetylation adds an additional negative charge onto the histone tails that neutralises the positive charge on the histones and decreases the interaction between histones and the negative charge on the DNA molecule. This causes a relaxing of chromatin structure and is associated with increased gene expression levels.

Alzheimers' disease

AD was first described by the psychiatrist Alois Alzheimer in Munich in 1906 (83). AD is the most common form of dementia, affecting up to 15 million individuals worldwide annually. Due to increase in life expectancy, by 2050 it is expected that approximately 25% of people living in the Western hemisphere will be over 65 years of age, one third of which are likely to develop AD (84). The high prevalence of the disease makes investigation on the pathogenesis and potential treatments of AD very important. AD is a neurodegenerative disease characterised by the formation of amyloid plaques and neurofibrillary tangles. Plaques consist of deposits of proteinaceaous material, a major component of which is β-amyloid. The neurofibrillary tangles are composed of paired helical filaments of the neuronal phosphoprotein tau.

Cholesterol and oxysterols in Alzheimer's disease

Many studies suggest that there is a link between cholesterol metabolism and AD (84). A hypercholesterolemic diet was shown to significantly increase β-amyloid load in rabbits by increasing both deposit number and size (85).

Recent studies show that the processing of APP is sensitive to cholesterol levels. Cleavage of APP by β-and γ-secretases produces Aβ. These cleavage events are more likely to occur in the high cholesterol environments of lipid raft domains (2). APP is a type-I glycoprotein with its amino terminus on the lumenal/extracellular surface and a short C-terminal cytoplasmic tail. The major component of amyloid plaques, the Aβ peptide (showed in figure 5), is produced by the β secretase pathway, where APP is

first cleaved at the N-terminus of Aβ (β-cleavage) and then in the transmembrane domain (γ-cleavage), either at position 40 or 42. In contrast, APP is more frequently cleaved at the α-position, between amino acids 16 and 17 of the Aβ region, precluding the generation of Aβ. The APP C-terminal fragments produced after α and β cleavage of APP are also respectively called C-83 and C-99, based on the number of amino acids (86). A study on a transgenic-mouse model for AD demonstrates that high dietary cholesterol increases Aβ-accumulation and accelerates the AD-related pathology (87).

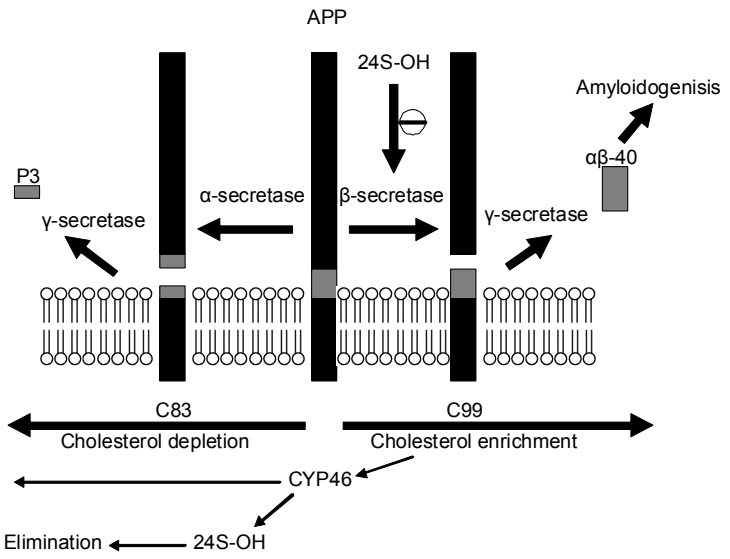


Figure 5. Amyloid precursor protein (APP) is a transmembrane protein that can undergo different cleavage pathways which result in either a soluble (P3) nonpathogenic or an insoluble pathogenic product (Aβ40). The activity of the enzyme CYP46 may affect the balance between α- and β- secretase activities as indicated.

Interestingly, polymorphisms in the CYP46A1 gene has been suggested to influence both Aβ peptide load in the brain and the genetic risk for late onset sporadic AD (LOAD). A single nucleotide polymorphism in intron 2 of CYP46 gene has been identified and reported to be significantly associated with increased risk for LOAD. According to the report, the frequency of the CYP46A1 T allele and TT genotype was significantly higher in AD patients from Switzerland, Greece, and Italy than in controls (88). It has also been reported that the CYP46A1 C allele might act as a risk factor for LOAD in Italian patients, meaning that the susceptibility polymorphism might differ in different ethnic groups (89). Recently, Kölsch *et al.* (90) identified two single nucleotide polymorphisms in CYP46A1 influencing AD risk and suggested that CYP46A1 gene variations might act as risk factors for AD via influence on brain cholesterol metabolism. During recent years the research about the relationship between polymorphism in CYP46A1 and AD has however given contradictory results and the most recent meta-analysis of the available data indicates no effect of the CYP46A1 polymorphisms. One possible reason could be the variability between different ethnic groups (91).

Our group has suggested that an upregulation of CYP46A1, resulting in an increased production of 24S-OHC may be neuroprotective and reduce formation of amyloid. One reason for this is the effect of 24S-OHC itself on amyloid formation as demonstrated in cultured neuroblastoma cells (92). Another reason is that increased consumption of cholesterol in the critical neuronal membranes as a consequence of increased activity of CYP46A1 would be predicted to reduce amyloid generation (Fig 5).

Levels of 24S-Hydroxycholesterol in AD

The possibility that altered plasma levels of 24S-OHC and other oxysterols may serve as markers of neurodegenerative processes such as AD has been extensively examined and assessed (93-97).

Reduced number of neuronal cells as a consequence of neurodegeneration would be expected to reduce the level of 24S-OHC in plasma.

Levels of 24S-OHC appear however to correlate with the stages of AD, with higher levels reported in early stages of the disease. This initial elevation may be due to an initial increased conversion of cholesterol to 24S-OHC due to increased substrate availability in connection with neurodegeneration and release of the cholesterol (95). This effect may be followed by decreased levels of 24S-OHC caused by loss of neuronal cells containing the CYP46A1 enzyme (94). Two studies have found induction of CYP46A1 in glial cells in the brain of AD patients, which suggests that reactive astroglial cells may be involved in turnover of cholesterol in AD brains (98, 99). The amounts of CYP46A1 in neuronal cells was decreased but was in part compensated by an induction of the enzyme in glial cells. The compensatory induction of CYP46A1 in astroglial cells may tend to counteract the decrease in plasma levels of 24S-OHC as a consequence of the neuronal loss.

It was also shown that the levels of 24S-OHC in CSF are elevated in AD patients indicating the possibility that CSF 24S-OHC may serve as a marker for neurodegeneration and may be used for monitoring the onset and progression of AD (100, 101). This possibility is the subject of *paper V*.

Fatty acids in the brain

Western diets are high in saturated fat and in combination with the sedentary lifestyles they have contributed to a growing incidence of obesity, hypercholesterolemia, and high blood pressure, causing atherosclerosis, coronary artery disease, and diabetes, major risk factors of AD (102). Fatty acids serve as both energy substrates and are essential for proper neuronal and brain function (102,103). With respect to the latter function polyunsaturated fatty acids (PUFA) are integral membrane lipids that serve to maintain both the structure and function of neuronal membranes, membranes that are associated with proteins and protein complexes.

The long-chain polyunsaturated fatty acids belong to two key families: omega-3 and omega-6, named after the position of the first double bond in the hydrocarbon chain counted from the methyl end. Docosahexaenoic acid (DHA) is the most abundant omega-3 fatty acid in the mammalian brain, which levels in brain membrane lipids can be altered by diet, and age (102, 104). DHA is considered to be an essential PUFA as *de novo* production is not possible in humans. Eicosapentanoic acid (EPA, 20:5n3) and α-linoleic acid (ALA, 18:3n3) can be converted to DHA by some cell types and organ systems in the body. There is however a great variability in the degree to which this can be accomplished in humans. While ALA can be derived from terrestrial plants and is

commonly found in a Western or terrestrial-based diet, DHA and EPA, initially created by photosynthetic microalgae, are almost exclusively derived from marine animals. Little EPA or ALA is found in the brains of humans, again attesting to the importance of DHA in the maintenance of neuronal membrane integrity and the signalling cascades related to PUFAs in the brain. According to a current concept, ratios of omega-3 to omega-6 PUFAs may be as important as absolute levels of these lipids in the homeostasis of CNS inflammation and oxidative stress (105). Very high levels of fatty acids and lipids can be found in the neuronal membrane and in the myelin sheath. About 50% of the lipids in neuronal membrane are composed of polyunsaturated fatty acids, while in the myelin sheath these lipids constitute about 70%. The integrity of the myelin is of utmost importance for the proper functions of axons in the nervous system. Breakage or lesions in the myelin can lead to disintegration of many of the nervous system functions. Recent studies emphasize the major role of dietary essential fatty acids to the normal functions of myelin. Moreover, the essential fatty acids are important in the active phase of the myelin synthesis. If essential fatty acids are not available in this phase or are metabolically blocked, amyelination, dysmyelination, or demyelination may occur (106). High DHA consumption is associated with reduced AD risk (102). A general consensus is that saturated fat should be reduced in our diet, whereas consumption of n-3 PUFAs should be increased. The recommendations vary widely among different countries as well as among nutritionists, and we still do not know the intake of n-3 PUFAs and the ratio of n-6 to n-3 PUFAs that would achieve optimum health benefits. The possibility that n-3 PUFAs are important for the activity of the CYP46A1 was studied in *paper III.*

Aims of the study

The following questions were asked:

- Is there a regulation of CYP46A1 at the epigenetic level?
- Is the drug Voriconazole also an inhibitor of CYP46A1 *in vivo*? Does the use of this drug cause changes in brain cholesterol homeostasis?
- Is there a dietary regulation of CYP46A1 by omega3 fatty acids?
- What are the metabolic consequences of an upregulation of CYP46A1?
- An interaction between 24S-OHC and APOE has been demonstrated *in vitro*. Is there a correlation between APOE and 24S-OHC in CSF?
- Can levels of 24S-OHC in CSF be used as a marker for neurodegeneration?

MATERIALS AND METHODS

The following is a summary of materials and methods used. A more detailed account is available in the respective papers. All experiments have been approved by the local ethical committee.

Paper I

Cell experiment- Neuroblastoma cells were exposed to TSA at a final concentration of 0.5 μM. After 0, 0.5, 1, 2, 4, 8 and 24 h of incubation the medium was removed and the cells were scraped into Trizol and stored.

Animal Experiment I—Long term effects of Valproate treatment. Six-week-old male C57/B6-J mice ($n = 5$) were injected intraperitoneally with 700 mg/kg Ergenyl (valproic acid). Controls ($n = 5$) received an equal volume of vehicle. Injections were continued for five days. On day six the animals were stunned with CO_2 and killed by cervical dislocation. Organs were removed and stored at -80˚C.

Animal Experiment II—Acute effect of TSA treatment. C57/B6-J mice were injected intraperitoneally with 0, 2, 5 and 10 mg/kg of TSA. Two hours after injection mice were stunned with CO_2 and killed by cervical dislocation. Organs were removed and stored.

Animal Experiment III—Long term effects of TSA treatment. Male C57/B6-J mice ($n = 5$) were injected intraperitoneally with 2 mg/kg TSA. Controls ($n = 5$) received an equal volume of vehicle. Injections were continued for nine days. On day nine the mice were stunned with CO_2 and killed by cervical dislocation. Organs were collected and stored.

Gene expression analysis. Total RNA was purified from cell or tissue samples using Trizol. cDNA was synthesised and steady-state mRNA levels were estimated using either Taqman probes or SYBR green chemistries. Cyclophilin A and hypoxanthine-guanine phosphoribosyl transferase (HPRT) were used as endogenous controls for cell culture and tissues.

Lipid extraction and analysis. Brain and liver tissue were homogenized according to Folch's method (107, 108). Extracts were dried under argon, redissolved in ethanol and stored at −20 °C until required. Sterols were analysed by gas chromatography–mass spectrometry (GC-MS).

Statistics. Gene expression data is expressed as mean ± range as described in Livak (109). Statistical comparisons were performed using the 2-tailed Students *t*-test, with the exception of the *in vivo* effects of histone deacetylase inhibitors on CYP46A1 expression. In accordance with our hypothesis that this gene is derepressed by these agents a one-tailed Student's *t*-test was used. A *P* value of less than 0.05 was considered significant.

Paper II

Voriconazole (vFEND®) was a generous gift from the department of pharmacology at Karolinska University Hospital in Huddinge. C57/B6 J mice were all seven weeks old males purchased from Charles River.

Animal experiment I- Acute Effects of Voriconazole - C57/B6 J mice (*n = 2*) were injected intraperitoneally (Ip) with Voriconazole (60mg/kg) and two control mice were injected Ip with 1% albumin solution. The mice were sacrificed by cervical dislocation after 6 hours. Organs were removed and stored.

Animal experiment II- Acute Effects of Voriconazole- As described above, C57/B6 J mice (*n = 2*) were injected Ip with Voriconazole but with 75mg/kg instead of 60mg/kg. The control mice *(n = 2)* were injected Ip with 1% albumin solution. The mice were sacrificed after 6 hours. Organs were removed and stored.

Animal experiment III- Acute Effects of Voriconazole- C57/B6 J mice (*n = 5*) were injected with Voriconazole (60mg/kg) as described previously. Control mice (*n = 5*) were injected Ip with 1% serum albumin. The mice were sacrificed 6 hours later. Organs were collected and stored.

Animal experiment IV- Long Term effects of Voriconazole- C57/B6 J mice (*n = 6*) were injected with Voriconazole (60mg/kg). Control mice (*n = 6*) received 1% serum albumin as described above. Injections were continued for 5days. On the fifth day the animals were sacrificed and organs and plasma were collected as described above.

Time course study- Effect of Voriconazole over time- C57/B6 J mice (*n = 6*) were injected with Voriconazole (60mg/kg). Control mice (*n = 6*) received 1% serum albumin. The treated animals and their respective controls were sacrificed as described above after 0, 2, 4, 6, 12, 24 hours. Organs and plasma were collected and stored.

Lipid Extraction
Lipids were extracted from the tissues as described in paper I according to Folch (cf. above).

Sterol analysis
Sterols were determined by isotope dilution–mass spectrometry (110). For determination of free cholesterol the samples were hydrolysed and [$^2H_6$] cholesterol was used as internal standard. [$^2H_3$] Lathosterol was used as internal standard for determination of lathosterol. The oxysterols, 24S-hydroxycholesterol and 27-hydroxycholesterol in the brain were analysed by GC-MS) (110).

Gene Expression Analysis- Total RNA was extracted from tissues .

Statistics-Gene expression data is expresses in mean ± range as described in paper I. Sterol determinations are presented as mean ± standard error of the mean (SEM). For statistical comparisons a two tailed student's T-test were preformed.

Paper III

Animals and Diets

Forty two 8-week-old male Golden Syrian hamsters (Harlan Sprague Dawley, Inc., Indianapolis, IN) were used in this experiment. After a initial 1-week adaptation period on a standard rodent diet AIN-93M (the diet of the American Institute of Nutrition for maintenance of mature rodents). 6 animals were sacrificed after overnight fasting (~16 hrs), and the other 36 animals were randomly assigned to three groups (n=12) and placed on experimental diets for 6 weeks. All diets contained cholesterol in the amount (0.025%) which is non-atherogenic for hamsters and equivalent to human intake of <300 mg/day. Palm oil, olive oil, safflower oil, and pharmaceutical grade fish oil were the major sources of SFAs, MUFAs, n-6 and n-3 PUFAs, respectively. Food and fresh water were provided ad libitum. Body weight was monitored weekly, and food intake daily at the end of the dark period. While all dietary groups had unrestricted access to food and always had some food left before a new portion of food was given to them, the total food consumption was reduced by 8% in group 3 fed the highest fish oil diet. This reduced food consumption led to a decreased by ~20% gain in the body weight in this group, however, did not seem to affect animal behavior or cause visible signs of malnutrition. After six weeks on custom diets, hamsters were deprived from food, and ~16 hrs later sacrificed in a chamber containing carbon dioxide. Blood was withdrawn immediately via cardiac puncture. Internal organs were excised, rinsed in cold 0.9% NaCl, blotted, cut into pieces, weighed, and flash-frozen in liquid nitrogen. All tissues were stored at -80^{o}C. The research was conducted in conformity with the Public Health Service Policy on Humane Care and Use of Laboratory Animals and approved by the University of Texas Medical Branch at Galveston Institutional Animal Care and Use Committee (protocol # 0707030).

Quantification of CYP46A1 protein in the brain

Total brain protein was separated by SDS-PAGE and transferred to a nitrocellulose membrane followed by the incubation with the rabbit polyclonal antibodies against human CYP46A1 (a generous gift from Dr. D Russell, UT Southwestern) and sheep anti-rabbit immunoglobulins conjugated with horseradish peroxidase. CYP46A1 and beta actin (used as a loading control) were detected. Chemiluminescent signals from both proteins were recorded and quantified and the CYP46A1/actin ratio calculated.

Quantification of sterols

24-Hydroxycholesterol, 27-hydroxycholesterol, lathosterol, cholesterol and 7-ketocholesterol in the brain were measured by GC-MS.

Paper IV (Manuscript)

Construction of Human CYP46 Overexpressor Transgenic Mice

Two 1,6-kb fragments (one included an HA-tag sequence) encoding for the human CYP46 cDNA was inserted into the EcoR1 restriction site in a pCAGGS expression vector (kindly provided by Prof. J Miyazaki). The pCAGGS vector contains the chicken β-actin promoter and rabbit β-globin poly(A) signal permitting an ubiquitous overexpression of the human CYP46 cDNA in all tissues. The microinjections were made by the core facility of Karolinska Institute (Karolinska Center for Transgene Technologies).

Genotyping of Transgenic Mice

The animals were tail clipped and genomic DNA was isolated The offspring were screened for the presence of transgenes by PCR analyses using specific primers. Positive transgenic mice were identified by tail DNA/PCR genotyping. PCR amplification was performed using primers specific to the human transgenic CYP46 sequence. The PCR resulted in a 193-bp fragment for the human cDNA transgene sequence.

Copy number

A method was established to evaluate the degree of overexpression among the positive transgenic mice using genomic tail DNA. Increasing amounts of genomic DNA were analysed by RT-PCR. One primer/set detected the human cDNA sequence of CYP46A1 whereas the other primer/set detected the mouse genomic Gpbar1 sequence. To estimate the amount of copies of the hCYP46A1, the slopes obtained by the regression of the Ct values and the amount of gDNA loaded in the reaction for the amplification of hCYP46A1 were divided by the slopes obtained from the amplification of the mGpbar1.

Back crossing

The mice with a HA-tag sequence were backcrossed with C57/Bl/6NCrl for 7 generations and then characterized. The mice without a HA-tag sequence were backcrossed for a few generations only before a preliminary characterization.

Western Blot Analysis

Microsomes prepared from brains, both transgenic and normal mice were subjected to electrophoresis in three different amounts and transferred to nitrocellulose membranes. The membranes were incubated for 2 hrs at room temperature in blocking buffer followed by incubation overnight in cold room with an anti-CYP46 antibody (a generous kind gift from Prof. D. Russell, University of Texas Southwestern Medical Center, Dallas,TX). As a secondary antibody, Goat-anti-rabbit coupled with horseradish peroxidase was used and incubated at room temperature for 2 hrs. In some experiments a human specific antibody: Rabbit polyclonal to CYP46 was used with Goat-anti-rabbit as a secondary antibody. A signal at (around 50 kDa) was detected. The results of the signal from each sample in triplicate were calculated as a linear value.

Lipid Extraction and Analysis

Lipids from brain and liver was extracted and analysed as described above.

Paper V and VI

Paper V- All patients included in the study were referred for cognitive impairment and suspected dementia from general practitioners in the greater Stockholm catchment area to the Memory Clinic at Karolinska University Hospital in Huddinge, Stockholm, during a period of 3 years. They underwent a comprehensive investigation including clinical examination, routine blood and CSF laboratory tests, electroencephalography (EEG), magnetic resonance imaging **(**MRI), and single photon emission computed tomography (SPECT). The cognitive evaluations included mini-mental state examination (MMSE) as well as comprehensive neuropsychological tests.

The 38 patients studied had a wide variation in the degree of cognitive impairment, from MCI to manifest AD with dementia, to reflect a continuum of cognitive impairment from mild to severe. The AD group included 18 patients, 10 men and 8 women, mean age 7 ± 4.2 years (range 69–85), MMSE 23.1 ± 5.4 (range 7–28). Alzheimer's disease was diagnosed according to the DSM-IV criteria (111). The MCI group included 20 patients, 10 men and 10 women, mean age 59 ± 10 (range 49–86), MMSE 28 ± 2.1 (range 22–30). The MCI diagnosis was assessed according to previous published criteria (112). All MCI subjects were referred for evaluation of cognitive impairment from general practitioners. The majority of the patients had memory problems, some of them had additional cognitive impairments. A minority had non-memory cognitive problems. Thus, this cohort represented a heterogeneous group of patients with several disorders explaining the cognitive impairment. The group is probably dominated by very-early Alzheimer's disease subjects but to what extent can only be established in a longitudinal follow-up study.

Paper VI- The patients included in the study were referred to Karolinska University Hospital in Huddinge, Stockholm. The MCI and AD groups were referred to the Memory Clinic from primary care centres in the catchment area for investigation of suspected dementia. These patients were all living independently in the community, i.e., they were not in need of formal care or aid from the community. They were evaluated according to a standard comprehensive protocol (113) including clinical examination, brain imaging MRI and SPECT, EEG, analyses of blood, urine and CSF (T-Tau, P-Tau, and Aβ42) and a detailed neuropsychological evaluation. Dementia and AD were diagnosed according to DSM-IV(55) and NINCDS-ADRDA (114) criteria. MCI patients were: (1) not demented; (2) had self and/or informant report of cognitive decline and impairment on objective cognitive tasks; (3) had preserved basic ADL/minimal impairment in complex instrumental functions (112). The control group included patients referred to the Neurology Clinic for headache of uncertain cause. They underwent a comprehensive clinical and laboratory evaluation which ruled out any signs of organic CNS disease.

Analysis of CSF - CSF was collected for diagnostic purposes by lumbar puncture. T-tau was determined using a sandwich enzyme-linked immunosorbent assay (ELISA) constructed to measure t-tau (both normal tau and p-tau). p-Tau (P-Thr181) was determined using a sandwich ELISA, with monoclonal antibody (MAb) HT7 (recognizing all forms of tau) used as capturing antibody and biotinylated MAb AT270 (specific to P-Thr181 p-tau) used as a detection antibody. $A\beta_{42}$ was determined using a sandwich ELISA specific for $A\beta_{42}$.

APOE levels in CSF were assayed by a slight modification of a commercial immunoassay for APOE in plasma. In the assay of APOE in CSF, undiluted CSF samples were analyzed in contrast to assay of plasma where dilutions 1:20 were used.

CSF levels of 24- and 27-OHC were assayed by isotope dilution-mass spectrometry a.

Statistical calculations- Analysis of variance and Student's test were used for group comparisons, while the Pearson correlation coefficient was used for correlations. The level of significance was set to $P = 0.05$.

RESULTS AND DISCUSSION

Paper I

Is there a regulation of cyp46a1 at the epigenetic level?

Derepression of CYP46A1 Expression by TSA

The mechanism(s) underscoring the ectopic expression of CYP46A1 has yet to be established. We thus investigated if CYP46A1 expression may be regulated by histone acetylation status. Treatment of SH-SY5Y neuroblastoma cells with TSA, led to a marked time dependent derepression of CYP46A1 expression which essentially restored the expression of CYP46A1 to that of neurons (Fig 6a). This observation was replicated in HepG2 hepatoma cells (results not shown). To further validate these findings we also performed RT-qPCR on SH-SY5Y cells treated with 0.5 μM TSA for 48 hours and observed a highly significant 150-fold increase in CYP46A1 expression ($P < 0.00001$) (Fig 6b). For reasons of comparison we also measured the expression levels of other genes known to be important for brain sterol homeostasis. There was a significant effect on the expression of CYP27A1 that increased about 5 fold, while the expression of CYP7B1 was decreased to about 10% of the control levels ($P < 0.0001$).

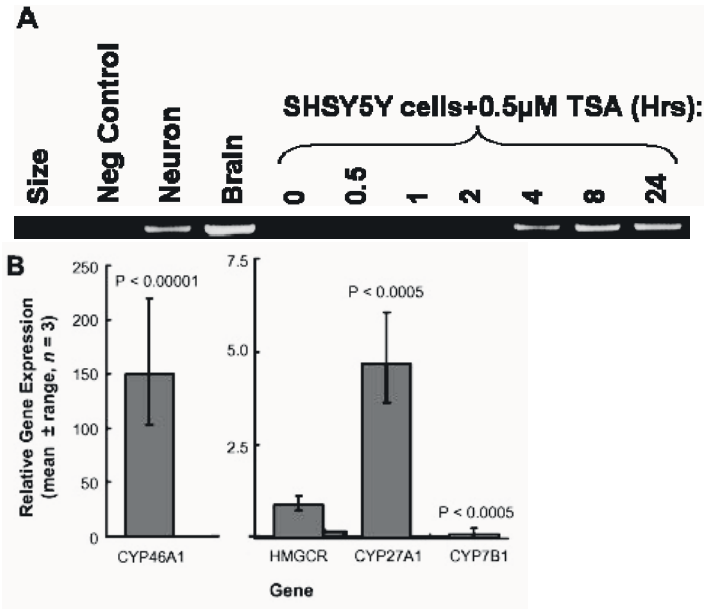


Figure 6. Derepression of cholesterol 24-hydroxylase by histone deacetylase inhibition. **A)** SH-SY5Y cells were treated with 0.5μM of TSA for the indicated time periods and gene expression was evaluated by reverse transcription-PCR; **B)** SH-SY5Y cells were treated with 0.5μM of TSA for 48 hours and relative gene expression was evaluated by quantitative reverse real-time PCR. HPRT was used as internal control.

Effects of Valproate Treatment on Cholesterol Homeostasis in mice

Given the potent effects of histone deacetylase inhibition on the mRNA levels of CYP46A1 under *in vitro* conditions, we considered it important to define if this effect could be replicated *in vivo*. Initially we used the anti-epilieptic and anti-anxiolytic drug valproic acid (VPA), which is well known to have histone deacetylase inhibitor activity. Intraperitoneal injection of a high dose of VPA (700mg/kg) resulted in a modest induction in the mRNA expression of Hmgcr and CYP46A1 in the liver, and CYP46A1 in the brain. It should be emphasized that the basal expression of CYP46A1 in the liver was very low. However, treatment with VPA at this level led to the death of three of the treated mice, two at day three and one at day four indicating that the observed changes in mRNA levels could have been influenced by the general toxic effects of VPA. A similar experiment with a lower dose of VPA (350 mg/kg body weight) led to a similar pattern of changes of mRNA with a significant increase in Hmgcr expression ($P < 0.05$) while there was a 1.5-fold increase in CYP46A1 ($P < 0.05$). There were no matching changes in hepatic or cerebral steady-state sterol levels. To exclude the possibilty that the capacity for downstream sterol metabolism (i.e. 7α-hydroxylation of 24S-OHC) may have been altered by VPA treatment, we measured the mRNA levels of Cyp39a1 in both the liver and brain. Surprisingly, we found a two-fold increase in expression of Cyp39a1 in the brain ($P<0.01$) and a 50% decrease in hepatic expression ($P < 0.002$) (Fig 7b).

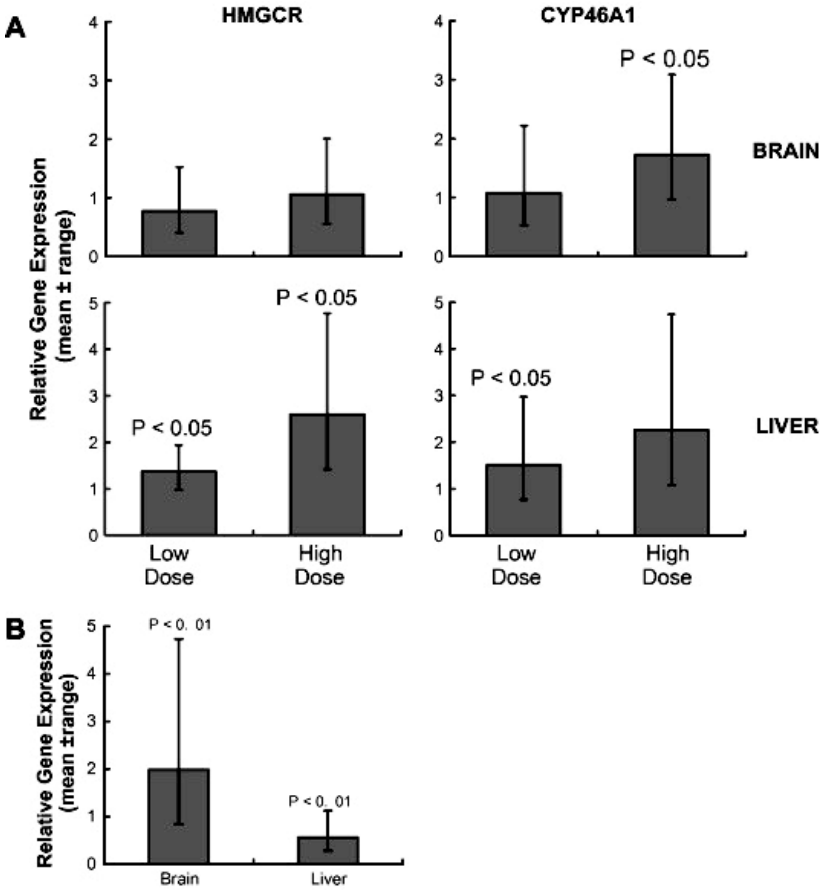


Figure 7. Effects of Valproate treatment on hepatic and cerebral gene expression in the mouse. Following intraperitoneal injection of low or high doses of Valproate, gene expression in the brain and liver was evaluated. **A)** Dose dependent effects on the expression of Hmgcr and CYP46A1. Treatment with Valproate led to modest dose dependent changes in the mRNA expression of Hmgcr and CYP46A1 in the liver, and CYP46A1 in the brain. **B)** Divergent regulation of Cyp39a1 expression in brain and liver. Treatment with Valproate lead to a two-fold increase in expression of Cyp39a1 in the brain ($P<0.01$), while hepatic levels were reduced to approximately half of the control value ($P < 0.002$) (Fig 7b).

Effects of TSA *Treatment on Cholesterol Homeostasis*

In light of the above mentioned toxic effects of high dose VPA treatment we decided to test the potent histone deacetylase inhibitor TSA. We initially studied the dose dependent effects of TSA treatment in an acute model. IP injection of TSA to male mice at doses from 2 mg/kg to 10 mg/kg body weight resulted in dose dependent increases in the expression of brain of CYP46A1 and Cyp39a1. This was recapitulated in the liver with increases in hepatic CYP46A1 and Cyp7b1 expression and decreases in both Hmgcr and Cyp39a1 levels (*Paper I).* Intriguingly, the response of female mice to the same regimen was markedly different (results not shown).

We then treated animals with 2 mg/kg TSA for nine days. This resulted in a small but significant reduction in brain Hmgcr mRNA expression ($P < 0.05$). No significant change was observed in the cerebral expression of CYP46A1, Cyp39a1 or Cyp7b1. The

situation in the liver was similar to that observed following VPA treatment, with a 2.5-fold increase in hepatic CYP46A1 expression. In accordance with our hypothesis that inhibition of histone deacetylase increases the mRNA levels of CYP46A1, this was significant following a one-tailed T-test ($P < 0.05$). Again, there were no differences in the brain or liver sterol levels.

The overall pattern of changes in the mRNA expression of CYP46A1 was remarkably consistent under a variety of different treatment schemes. The evaluation of the results are complicated by the pharmacokinetic profile of TSA – the plasma half life of this compound in mice is in the order of 7-9 minutes and available data indicates significant and rapid hepatic metabolism, potentially to less active compounds (115). The short half life may be part of the reason for the differences between the acute and chronic TSA mediated regulation of selected genes e.g. Cyp7b1.

An intriguing finding of our investigations on the acute effects of TSA was the finding of gender specific responses to effects of the treatment. It has been established that hepatic expression of Cyp7b1 is greater in male mice, whereas that of Cyp39a1 is greater in females (116). This pattern appears to be potentiated by TSA treatment, at least under acute conditions, suggesting that epigenetic mechanisms may be a general regulator of sexually dimorphic cytochrome P450 expression.

A surprising finding was the detection of significant levels of Cyp39a1 in the brain. This cytochrome P450, which is a 7α-hydroxylase specific for 24S-OHC, was originally cloned as a liver specific species (116,). Subsequent investigations have identified Cyp39a1 mRNA or protein in brain or the non-pigmented epithelium of the retina (117). At the current time, the reason for the diametrically opposite regulation in brain and liver is unclear. Expression in the brain may provide a plausible explanation for absence of changes in brain 24S-hydroxycholesterol - induction of the 24S-OHC 7α-hydroxylase within the brain may theoretically lead to intracerebral production of 7α, 24S-dihydroxycholesterol and mask any increase in 24S-OHC content. Attempts to identify the latter steroid in mouse brain has failed thus far, however.

It has previously been shown that epigenetic mechanisms may be involved in the regulation of sterol synthesis – Villagra *et al.* demonstrated that lanosterol synthase is repressed by histone deacetylase-3 and presented data that some other genes in the cholesterol synthesis pathway were regulated by histone deacetylase inhibition (118). The current data supports the contention that epigenetic mechanisms may be of importance for the regulation of the transcription of several cytochrome P450s involved in metabolism and elimination of cholesterol.

Paper II

Is the drug Voriconazole an inhibitor of cyp46a1 also *in vivo*? Does the use of this drug cause changes in brain cholesterol homeostasis?

Inhibition of cholesterol 24S-hydroxylase activity in vitro

In the present study we tested four antifungal azoles that are used systemically as therapeutic agents with respect to binding to CYP46A1.We established that Voriconazole binds with high affinity to CYP46A1 in vitro and efficiently inhibits CYP46A1 -catalyzed cholesterol 24-hydroxylation in the reconstituted system. These azoles are interesting because of the fact that they are known to pass the BBB.

When added to full-length human CYP46A1 Voriconazole caused a Type II spectral response with a minimum at 412 nm and a maximum at 431 nm (experiments performed in the laboratory of Prof. Pikuleva). The spectral Kd was found to be 0.26 μM and 0.05 μM when Voriconazole was added in methanol and 50% methanol respectively (Paper II). Under the conditions employed with 2.7 μM cholesterol and 43 μM Voricoanzole the inhibition of cholesterol hydroxylation was 91%.

When tested at a cholesterol concentration equal to the Km, the Ki value was found to be 11 $\pm$3 nM. For reasons of comparison Voriconazole was also added to a preparation of full length bovine CYP11A1. Also in this case there was a Type II spectral response with a spectral Kd of 6.5 μM (*Paper II*). When added to full-length human CYP27A1 and to truncated CYP7A1 under the same conditions, there was a weak spectral response only.

Inhibition of cholesterol 24S-hydroxylase in vivo

In a pilot study we exposed mice to different doses of Voriconazole, killed the mice and their controls six hours after injection and measured the levels of 24S-OHC in the brain. The treatment appeared to reduce the levels of 24S-OHC. The levels were slightly lower after injection with 60 mg/kg body weight than after injection with 75 mg/kg boy weight (results not shown). In a subsequent experiment we treated 5 mice with Voriconazole, 60 mg/kg body weight as above and killed the animals and their controls after 2 hours. The levels of 24S-OHC were reduced by 20% but this effect was not statistically significant ($p>0.05$, Student´s t-test). There was no significant effect on levels of lathosterol or total cholesterol in the brain (results not shown).

The result of an experiment where 6 mice were treated with Voriconazole, 60 mg/kg body weight, once daily for 5 days, indicate that there was a significant reduction in the levels of 24S-OHC, by about 20 % (Fig. 8). There was no effect on levels of cholesterol or 27-OHC *(Paper II).* The ratio between lathosterol and cholesterol in the brain was significantly reduced, however, indicating a reduced cholesterol synthesis ($p<0.05$).

Since most of the 24S-OHC present in the circulation originates from the brain, a reduction of brain synthesis is likely to lead to a reduction of plasma 24S-OHC, unless Voriconazole has an effect on the metabolism in the liver. A pool of plasma from the Voriconazole-treated mice had a concentration of 24S-OHC of 13 ng/mL. A pool of plasma from the control mice had a concentration of 24S-OHC of 19 ng/mL. Thus Voriconazole appeared to suppress the plasma levels by about 30%. It should be pointed out that the level of the 27-OHC was not significantly affected by the

Voriconazole treatment. The levels of this oxysterol was found to be 46 ng/mL and 52 ng/mL in the Voriconazole treated mice and in the controls, respectively.

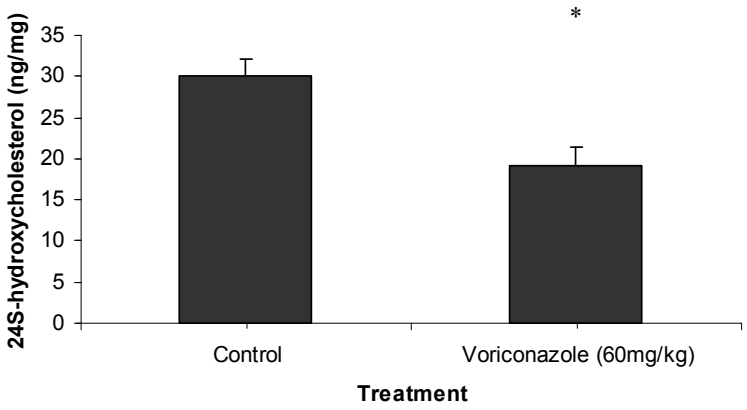


Figure 8. 24S-OHC measured in mice brain after daily injection of Voriconazole. Values are expressed mean ± SEM. * = Statistical significant at (P = 0,003) as compared to control.

Effects of Voriconazole on the levels of cholesterol precursors upstream of lathosterol

The reduced levels of lathosterol in the Voriconazole-treated mice suggest a reduced cholesterol synthesis. Part of this reduction could be due to the inhibition of cyp51, the enzyme responsible for demethylation of lanosterol, an upstream precursor of lathosterol. In accordance with this, we found that the brain levels of lanosterol increased from 6±2 ng/mg tissue in the controls to 27±10 ng/mg tissue in the Voriconazole-treated mice ($p<0.001$). The levels of dehydrolanosterol were 0.14±0.05 ng/mg tissue and 2.0±0.7 ng/mg tissue, respectively. The levels of the lanosterol precursor squalene were 2.2±0.8 ng/mg tissue in the controls and 1.7±0.6 ng/mg tissue in the Voriconazole-treated mice ($p=0.05$). The latter is consistent with a reduction of cholesterol synthesis also at a step prior to lanosterol demethylation.

Effects of Voriconazole on mRNA levels of genes involved in cholesterol homeostasis

Voriconazole had no significant effect on expression of CYP46A1 mRNA or Hmgcs *(Paper II).* There was a significant suppressive effect on Hmgcr mRNA levels, however (*Paper II).*

Brain levels of Voriconazole

The level of Voriconazole in the brains of mice (n=5) after 5 daily intraperitoneal injections (60 mg/kg body weight) was 43 + 8 μg/g wet weight corresponding to 123 μM. Kinetics of brain levels of Voriconazole after a single injection (60 mg/kg) is shown in Fig. 9.

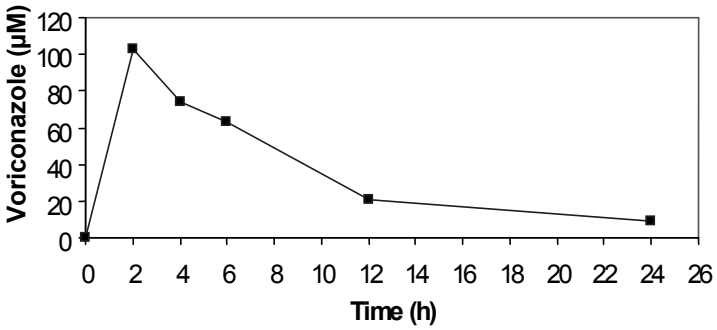


Figure 9. Kinetics of brain levels of Voriconazole in mice after a single intraperitoneal injection (60 mg/kg body weight).

As would have been expected from the structure of the binding site of CYP46A1, Voriconazole was found to be an efficient inhibitor of cholesterol 24S-hydroxylase activity in vitro.
In accordance with this, and in accordance with the fact that Voriconazole is known to pass the BBB, a statistically significant decrease in 24S-OHC levels could be demonstrated in the brains of mice injected intraperitoneally with Voriconazole for 5 days.

In theory, there are two possible explanations for the reduced levels of 24S-OHC in the brains of the treated animals. The first explanation is a direct inhibition of the enzyme by Voriconazole. The very high levels of Voriconazole measured in the brain in relation to Ki are in accord with this. The second possibility is related to the fact that Voriconazole is an inhibitor of cholesterol synthesis. It has been shown that Voriconazole exerts its antifungal effect through inhibition of the cytochrome P-450 enzyme 14 alpha sterol demethylase, CYP51, an enzyme responsible for the demethylation of lanosterol in the ergosterol biosynthesis pathway (119). Theoretically a reduced synthesis of brain cholesterol may eventually lead to reduced substrate availability for the CYP46A1 enzyme with reduced production of 24S-hydroxycholesterol.

The reduced levelsof lathosterol in relation to cholesterol indicates that brain cholesterol synthesis was reduced in the Voriconazole treated mice. This is consistent with both the above mechanisms. The fact that the Voriconazole treatment had no significant effect on the pool of cholesterol in the brain, does not favour the hypothesis that reduced substrate availability is of importance for the reduced production of 24S-hydroxycholesterol. However, the cholesterol content in some specific brain cholesterol pools may have been influenced. Our studies on the whole brain would not be able to resolve this issue.
A primary effect of Voriconazole on CYP46A1 can be expected to lead to reduced consumption of brain cholesterol and thus a reduced need for de novo synthesis. The finding that the Voriconazol treatment had a suppressive effect on the expression of

Hmgcr mRNA is consistent with this explanation. In this connection it is of interest that a complete gene knock-out of CYP46A1 in a mouse model is associated with a reduction of cholesterol synthesis by about 40% (120).

Treatment with Voriconazole represents a less drastic experimental model for inhibition of the enzyme than the knock-out model. Voriconazole may thus be used for detailed studies on the relation between CYP46A1 activity and cholesterol homeostasis. In such studies it is necessary to define the degree of inhibition of the enzyme by measurement of the product 24S-OHC in the brain.

One of the most important side-effects of Voriconazole is visual disturbances. Since the neuronal part of the retina contains even higher concentrations of CYP46A1 than the brain (121), the possibility may be considered that the visual disturbances in connection with Voriconazole treatment are related to an interaction between the drug and retinal CYP46A1. If this is the case, visual disturbances would be expected in CYP46A1 gene knockout mice. In accordance with this contention, abnormalities in the electroretinography (ERG) signals have recently been demonstrated in such mice (D.Russell, personal communication). Further work on a possible relation between vision and cholesterol 24S-hydroxylase is in progress.

Paper III
Is there a dietary regulation of cyp46a1 by omega-3 fatty acids?

Choice of animal model and diet
In this study we chose hamsters since they represent a better animal model for the evaluation of the effect of dietary cholesterol and n-3 PUFAs than mice and rats. Also, unlike previous animal studies, in which n-3 PUFAs were added to the standard rodent chow (122-125), we used "human-like" diets.

Serum lipids

The baseline levels of serum lipids were determined prior to testing the effect of diets. Measurements were carried out after a 1-week adaptation period during which the animals were fed regular rodent diet AIN-93M. Mean T-C (103 mg/dL), LDL-C (61mg/dL), HDL-C (48 mg/dL), and TG (175 mg/dL) were at or around the levels suggested as desirable in humans by the American Heart Association (<200 mg/dL T-C, <100 mg/dL LDL-C, >40mg/dL HDL-C, and <150 mg/dL TG). Switching to the "human"-like diets 1-3 led to an increase in T-C, HDL-C and TG and a change in the LDL-C to HDL-C ratio. This result is consistent with the previous findings by others showing that unlike rats and mice, which are highly resistant to the effects of dietary cholesterol, hamsters are moderately responsive, and thus represent a better model for nutritional studies.

The AIN-93M diet was cholesterol-free and only 9% of calories were from fat, whereas diets 1-3 contained 0.025% cholesterol and fat provided 34% of calories. Changes in the levels of serum lipids were also observed between the diets. Increased intake of the fish oil led to a decrease in the mean levels of LDL-C, HDL-C, and TG in diets 2 and 3 but only in the highest fish oil group (diet 3) did this decrease reached to a statistical significance.

Similar to humans, hamsters in our study had a decrease in TG levels upon increased intake of n-3 PUFAs; this decrease, however, did not reach a statistical significance. The effect on LDL-C and HDL-C was the opposite to that seen in many, but not all, human trials; both lipoproteins were significantly lower in hamsters on diet 3 containing the highest amount of n-3 PUFAs but the LDL-C/HDL-C ratio did not seem to change in the three test groups.

Expression of genes for Cyp7a1, Cyp27a1, and CYP46A1

Cyp7A1 mRNA levels, showed a significant,(~ 17- to 44-fold), intergroup variability, Cyp27a1 a moderate, (4-6-fold), intergroup variability, and CYP46A1 a low, only (2-3-fold), intergroup variability. Because of this high variability, a decrease in Cyp7a1 in groups 2 and 3 was not statistically significant. Cyp27a1 and CYP46A1, however, did show a statistically significant increase in group 3 in the liver and brain, respectively.
The high intergroup variability of Cyp7a1, was present at all levels; mRNA, protein and enzyme activity. This result is consistent with the previous findings in humans demonstrating that CYP7A1 activity varied markedly, over a 5-10- fold range in healthy individuals (126, 127-129) It is likely that it is effects on Cyp7a1 transcription that determine different mRNA levels and consequently enzyme activity. In hamsters and humans, the gene transcription is not sensitive to cholesterol but is suppressed by bile acids (130-134). Transcription factors that negatively regulate Cyp7a1 (the farnesoid X receptor, fibroblast growth factors 4 and 15 and the protein β-Klotho (135)

are responsive to different stimuli, and this sensitivity to different stimuli may explain interindividual variability in the human gene expression as well as our data. Diurnal rhythm is also of importance for the activity of Cyp7a1 (136,137). Despite the carefully controlled conditions in our study, hamsters still had a marked intergroup variability suggesting factors other than age, sex, health status and diet to affect Cyp7a1 transcription. One of such factors could be starvation or individual differences in the time of the last food intake. Both of these factors were reported to affect the Cyp7a1 mRNA levels (136). Another factor could be genetic differences.

Since CYP27A1 and CYP46A1 are also expressed in the retina (121) we measured Cyp27a1 and CYP46A1 levels in the eyeball as well. In these experiments we combined three eyeballs, each from a different animal, to obtain one mRNA sample, and, thus, had four mRNA samples per group. No differences in abundance of Cyp27a1 and CYP46A1 in the eyeball were found between the three dietary groups.

Levels of sterol markers for cholesterol synthesis and degradation in the brain

Of the three sterols measured in the brain, lathosterol, 24S-OHC, and cholesterol, the former showed the highest intergroup variability (up to 6.7-fold), 24S-OHC varied up to 2 -fold and cholesterol only up to 1.5-fold. Increased fish oil content in diet 3 appeared to have a slight stimulatory effect on brain levels of 24S-OHC, 27-OHC and lathosterol. All these effects were however not significant from a statistical point of view.

Cyp46a1 protein expression in the brain was assessed by comparing the signals from Cyp46a1 and actin on Western blots. The Cyp46a1/actin ratio was slightly increased in diet 3 but this increase was not statistically significant and was not translated into increased serum 24S-OHC levels. Thus, diet 3 had an effect on the CYP46A1 mRNA levels in the brain and a trend to increased cerebral cholesterol turnover, yet changes in the levels of either Cyp46a1 protein or sterol markers were not significant.

Cyp27a1 is also expressed in the brain (138, 139), and thus we determined brain levels of 27-OHC. In both dietary groups they were more than 170-times lower than those of 24S-OHC. Thus, similar to humans and mice 24S-OHC also appears to be the major route for enzymatic cholesterol elimination from the brain in hamsters. The mean levels and the 27-OHC to cholesterol ratio were slightly higher in group 3 than in group 1. The intergroup variability in 27-OHC in the brain was up to 2-fold (not shown).

Increased n-3 PUFAs consumption was found to be positively associated with increased oxidative stress in several studies (reviewed in 141). Therefore, we measured 7-ketocholesterol, a toxic oxysterol, formed non-enzymatically from cholesterol. There was only a slight increase in this oxysterol level in group 3.

In our study we detected a clear effect of n-3 PUFAs on the Cyp46a1 mRNA levels in the brain of hamsters in group 3, and this effect is of interest in relation to a previous failure to demonstrate enzyme regulation at the transcriptional level by a number of hormonal and dietary factors (73). The protein and 24S-OHC levels in group 3, however, were not significantly increased, and serum 24S-OHC levels were unchanged. It is possible that there is a time lag between the mRNA and protein synthesis and consequently the production of 24S-OHC. There was a tendency to increased cholesterol synthesis but this effect was not statistically significant in group 3.

Studies in mice indicate that cholesterol biosynthesis and elimination are tightly coordinated in the brain (140). Mice lacking Cyp46a1 have decreased cholesterol elimination from the brain and a compensatory decrease in cholesterol biosynthesis (80). The steady state cerebral cholesterol levels in Cyp46a1 knockout mice remain the same as in wild type animals but cholesterol turnover is decreased leading to deficiencies in memory and learning (80). The consequences of the increased cholesterol elimination from the brain were investigated in a recent gene therapy study (78). Transgenic mice representing a model of AD were transfected with CYP46A1 cDNA in hippocampus and cortex using an adeno associated virus approach (143). The treated animals had increased levels of 24S-OHC in the brain but unchanged cholesterol levels suggesting increased cerebral cholesterol biosynthesis and turnover. These animals showed improved cognitive performance and also had a reduced amyloid plaque formation (78). Thus, although we could not demonstrate a statistical increase in the cerebral cholesterol turnover in hamsters on diet 3, the observed changes are in the direction expected for increased Cyp46a1 mRNA level and consistent with the results of many studies indicating that fish consumption or intake of n-3 PUFAs beneficial effects on brain function (reviewed in (144)). It should be noted that our data does not exclude a possible difference at the ± 10-20% level.

In summary, our nutritional studies in hamsters indicate that Cyp7a1 gene transcription contributes to significant interindividual variations in CYP7A1 protein levels and enzyme activity and the latter could underlie the different effect of n-3 PUFAs on serum cholesterol and inconsistent results of human trials. The results are consistent with the possibility that increased dietary intake of n-3 PUFAs may lead to increased activity of Cyp46a1 and a stimulation of cholesterol turnover in the brain.

Paper IV (Manuscript)

What are the metabolic consequences of an upregulation of cyp46a1?

Characterization of CYP46A1 overexpressing mice

The transgenic mice behaved normally and there was no difference in weight, female/male ratio or reproduction between these mice and their non-transgenic littermates. No abnormalities were observed in the brain, liver or adrenals. Significant levels of mRNA corresponding to human CYP46A1 were detected in brain, liver, ovary, testes, eye, kidney and lung. CYP46A1 protein could only be detected in brain, testis and the eye. However CYP46A1 protein levels in eye and testis of the overexpressed animals appeared to be less than 10% of those in the brain.
The levels of 24S-OHC were significantly increased in the testis, eye and in the brain of the overexpressed mice as compared to those of the wildtype. The levels of 24S-OHC in testis and eye were however less than 3% of those in brain. The plasma levels of 24S-OHC were increased about 4.5-fold in overexpressed female mice as compared to wildtype females. The corresponding figure for male mice was about 6.5-fold. The fecal excretion of free 24S-OHC was increased more than 20-fold in both overexpressed males and females. Immunochemical analysis was made on brain of overexpressed and control mice, using antibodies reacting with both murine CYP46A1 and human CYP46A1. Similar staining was obtained in the neuronal cells of the cortex, cerebellum and hippocampus. The pattern was identical in the transgenic and the wildtype mice with a tendency to stronger staining in the transgenic mice. In both cases expression was only observed in the neuronal cells, with no significant expression in the glial cells.

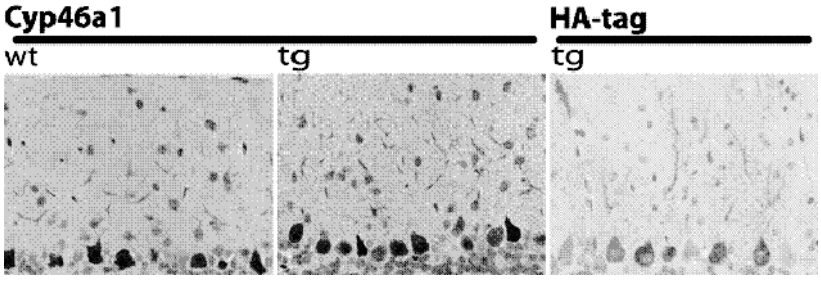


Fig 10. Immunohistochemical analysis of cerebellum (purkinje cells) from control and overexpressed mice. The primary antibody used was active towards both mouse and human *CYP46A1*. The right panel shows immunohistochemical analysis of the same region of the brain of an overexpressing mouse using antibodies directed towards the HA tag. For experimental details and the picture in colour see *paper IV*.

Western blotting of electrophoretically separated microsomal brain protein from overexpressing and control mice using antibodies active towards both human and murine CYP46A1 showed a 2-4 fold increase of CYP46A1 protein as a consequence of the overexpression (Fig 11).

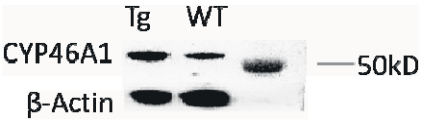


Fig 11. Western Blott analysis of CYP46A1 in transgenic mice compared to wildtype mouse.

The brain levels of 24S-OHC were increased almost two-fold both in the male and in the female overexpressing mice. There was a high correlation between the levels of 24S-OHC in the brain and in the circulation. High correlations were also observed between mRNA levels of human CYP46A1 and levels of 24S-hydroxycholesterol in the brain and in plasma.

Overexpression of CYP46A1 would be expected to consume cholesterol and result in a compensatory increase in cholesterol synthesis. In accordance with this the brain levels of a number of cholesterol precursors: 7-dehydrocholesterol, lathosterol, lanosterol, dihydrolanosterol, and FF-mas were all increased in the overexpressed mice. The levels of T-mas was slightly lower in the brain of the overexpressed mice. The levels of desmosterol in the brain of the overexpressed mice were not significantly higher than those of the controls. The levels of cholesterol were not significantly changes in the brain of the overexpressing mice.

The mRNA level of Hmgcr was slightly increased whereas Hmgcs mRNA in the brain was slightly decreased. The mRNA level of Cyp27a1 was significantly decreased. The mRNA levels of Srebp1c, Srebp2, Abca1, Cyp7b1, Cyp39a1 and Fas were unaffected by the overexpression of CYP46A1. There was no gender difference between males and females. LXR target genes were also measured in the liver (Cyp7a1, Cyp7b1, Srebp1c, Fas, Abca1, Abcg5, Abcg8). LXR-activation would be expected to increase expression of all the above genes, with the exception of Cyp7b1 that would be expected to decrease (144). There were marked gender differences in the expression of some of these genes. There was a tendency to a decrease in the expression of Cyp7b1 (not statistically significant) in the liver of females whereas it was significantly increased in males. Instead of an expected increase there was a significant decrease in the expression of the LXR target genes Srebp1, Cyp7a1, Abcg5, and Fas in the liver of the male CYP46A1 overexpressing mice. A similar effect was not seen in the liver of the female CYP46A1 overexpressing mice.

In addition to 24S-OHC, 27-OHC is a major oxysterol in the circulation. Surprisingly, the level of this oxysterol was significantly increased both in the brain and in plasma of the overexpresed mice. The mRNA level of Cyp27a1, the enzyme responsible for formation of 27-OHC, was decreased rather than increased in the brain.
A possible mechanism behind the increased levels of 27-OHC in the overexpressed mice could be a direct inhibition of the oxysterol 7α-hydroxylase by 24S-OHC. In order to study this possibility we incubated pig liver microsomes containing Cyp7b1 activity together with mixtures of 27-OHC and 24S-OHC. When incubating liver microsomes with 27-OHC at a saturating concentration together with a 4-fold excess of 24S-OHC, the addition of 24S-OHC caused an inhibition by more than 50% of the rate of 7α-hydroxylation of 27-OHC. Similar degree of inhibition was also obtained at lower substrate concentration. According to a Lineweaver–Burk plot the inhibition appeared to be of competitive nature.

The use of a ubiquitous expression vector caused expression of human CYP46A1 mRNA in several different organs with the highest expression in the brain, lung and testes. The expression caused a 4-6 fold increase in the level of 24S-OHC in plasma and a more than 20-fold increase in the excretion of this oxysterol in faeces. In view of the many different possible metabolic pathways for 24S-OHC: sulfatation, glucuronidation, omega-oxidation and conversion into bile acids (145), the increase in fecal excretion of the free steroid may be higher than the true increase in over-all production of 24S-OHC. On the other hand it is possible that the relatively moderate increase of 24S-OHC in the brain does not adequately reflect the total production and flux of 24S-OHC. It is important to emphasize that it may be the rate of the flux of the oxysterols rather than their absolute concentration that may be of importance for their biological effects. The relatively low levels of 24S-OHC may be due to an increased metabolism. Attempts to quantitate the major primary metabolite of 24S-OHC, 7α, 24-dihydroxycholesterol, failed, however. mRNA corresponding to the oxysterol 7α-hydroxylase Cyp39a1 were not increased as a consequence of the overexpression of CYP46A1.

Except for the brain, testis and the eye, the levels of CYP46A1 protein were low or undetectable in the different tissues tested, both in wildtype and overexpressed mice. This was the case also with the levels of 24S-OHC. Interestingly, next to the brain, the highest levels of 24S-OHC were observed in testis and eye, possibly in part due to the presence of a blood-testis barrier and a blood-retina barrier. We have previously shown that the blood-testis barrier has a preventive effect on the flux of lipoprotein-bound cholesterol from the circulation into testis (147).

Under normal conditions CYP46A1 is located almost exclusively to the brain in mammals, and most or all 24S-OHC originates from this organ. We have shown, however, that part of the 24S-OHC present in the circulation of mice may originate from other sources than the brain (147). Since there are significant levels of 24S-OHC in the circulation of mice homozygous for an induced null allele in the CYP46A1 gene (120), there must be small amounts of an unidentified enzyme in this species capable to introduce a hydroxyl group in the 24-position of cholesterol. How much of the increase in circulating levels of 24S-OHC that originates from the brain in the present mouse model can not be determined with certainty. In view of the very low levels of CYP46A1 protein in any other organs than the brain, it seems likely that most of the 24S-OHC in the circulation originates from the brain.

A more detailed investigation with respect to cellular location of the enzyme was made only in the brain. The immunohistochemical analysis revealed that the expression of the human CYP46A1 transgene was similar to that of the endogenous murine CYP46A1, and only neuronal cells were found to contain the expressed protein.

The overexpression of CYP46A1 would be expected to lead to a local consumption of cholesterol in the cells expressing CYP46A1 with a compensatory increase of cholesterol synthesis. In accordance with this, we found significantly increased levels of lathosterol and other cholesterol precursors in the brain of the overexpressed mice. There was a slight increase in the levels of mRNA corresponding to the rate-limiting enzyme in cholesterol synthesis, Hmgcr. A slight but significant effect on Hmgcr mRNA level was observed in hippocampus and cortex also in the study by Hurdy *et al.* (143). The total brain cholesterol levels were not significantly affected by the overexpression of CYP46A1 in the present work. It should be pointed out that it was

the total cholesterol that was measured, including both myelin cholesterol and cholesterol in neuronal and glial cells. In the study by Hurdy *et al.* (143), global cholesterol in hippocampus and cortex was unaffected by the overexpression.

In view of the increased levels of 24S-OHC in the circulation and tissues of the overexpressed mice, a general activation of LXR and LXR-targeted genes would be expected. There was however no significant activation of such genes, neither in the brain, nor in the liver. It can be concluded that 24S-hydroxycholesterol is less important as a general regulator of LXR-targeted genes. An LXR agonist has been shown to have a beneficial effect on neurodegeneration (148), and in view of the efficient binding of 24S-OHC to LXR it is tempting to suggest that this oxysterol could act by the same mechanism. The present work does not, however, suggest that an increase in the expression of 24S-OHC could have a beneficial effect on neurodegeneration by activating LXR. A similar conclusion was drawn from the results obtained with the mouse model used by Hudry *et al.* (143).

A surprising finding in the present study was that overexpression of CYP46A1 increased the levels of 27-OHC in brain and in circulation. We have shown that most of the 27-OHC present in the brain of humans originates from the circulation (149), and it is likely that this is the case also in mice. The increased levels of 27-OHC were not caused by increased expression of the enzyme producing this oxysterol, since Cyp27a1 mRNA levels were not increased by the overexpression of CYP46A1, neither in the liver, nor in the brain. Another theoretical possibility could be an effect on the enzyme responsible for metabolism of 27-OHC Cyp7b1. The expression of this enzyme is reduced by LXR-activation (145, 150). The mRNA levels corresponding to Cyp7b1 were however not affected by the overexpression of CYP46A1 in the brain, and in the liver different effects were obtained in males and females. Cyp7b1 is not active towards 24S-OHC, but it was shown that 24S-OHC can function as a competitive inhibitor of Cyp7b1 at concentrations similar to those in the circulation. We suggest that the increased levels of 24S-OHC may have a direct inhibitory effect on Cyp7b1 activity resulting in increased levels of 27-OHC in the circulation. This is of interest in relation to the previously unexplained findings of a close correlation between levels of 24S-OHC and 27-OHC in CSF of humans under different conditions (149, 100). In addition to an effect of 24S-OHC on the activity of Cyp7b1 it seems likely that this oxysterol could compete with 27-OHC for other excreteory pathways such as glucuronidation, sulfatation or conversion into bile acids.

It is concluded that our mouse model with overexpressed human CYP46A1 is possible to use for studies on a possible protective effect of its activity on neurodegeneration and amyloid accumulation in the brain. Work on this is in progress.

Paper V

Is there a correlation between APOE and 24S-OHC in cerebrospinal fluid?

In control patients there was a significant correlation between the APOE levels and cholesterol but no significant correlation with 24S-OHC or 27-OHC in CSF (Fig. 12A). The APOE levels were not correlated to $Albumin_{CSF}/Albumin_{plasma}$ ratio (QAlb) or CSF albumin. Age was not significantly correlated with 24S-OHC, ApoE, CSF Albumin, Cholesterol or 27-OHC. In accordance with the previous finding that the levels of 27-OHC in CSF are dependent upon the function of the BBB. There was a correlation between 27-OHC and QAlb *(paper V)*.

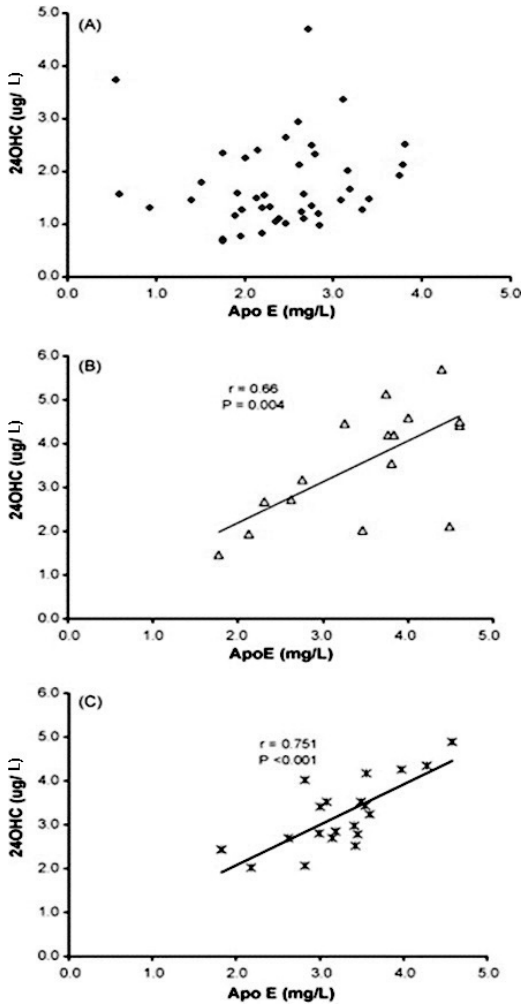


Figure. 12. Relation between APOE and 24S-OHC in cerebrospinal fluid from controls **(panel A)**($r = 0.1$, $p = 0.53$), AD patients **(panel B)** ($r = 0.66$, $p = 0.004$) and MCI patients **(panel C)** ($r = 0.75$, $p < 0.001$).

In AD-patients the APOE levels were not significantly correlated to cholesterol but there was a highly significant correlation to levels of 24S-OHC (Fig.12 B). There was no significant correlation to levels of 27-OHC. In similarity with the situation in

control patients there was no significant correlation between *APOE* levels, QAlb or CSF albumin. Also in this case the age was not correlated with any of the CSF variables studied.

In MCI patients the APOE levels were not significantly correlated to cholesterol but there was a highly significant correlation to levels of 24S-OHC (Fig. 12 C). There was no significant correlation to 27-OHC, Albumin or QAlb ($r = 0,07$, $p = 0,6$ ns). Age was not correlated with any of the CSF variables studied.

We compared APOE levels in CSF with other markers for neurodegeneration such as Tau levels in CSF from AD and MCI patients. In the AD patients APOE was found to be significantly correlated with Tau and P-Tau proteins but not with the $A\beta_{1-42}$. In the case of MCI patients the APOE levels were not significantly correlated with Tau and P-Tau, but significantly with $A\beta_{1-42}$.

Next we compared the controls with MCI and AD in order to evaluate the significance of the detected differences. The AD patients were significantly older than the controls and the MCI patients and the latter group was slightly but significantly older than controls. With respect to the APOE levels, these were significantly higher in the AD and MCI patients than in the controls. The levels of 24S-OHC and 27-OHC were significantly higher in AD and MCI patients compared to controls, as reported in paper VI. There was no significant difference between the three groups with respect to the levels of cholesterol, CSF albumin or QAlb.

Due to the significant age difference we evaluated the differences observed after age-correction, even if there was no significant correlation with age for any of the variables. After correction for age the levels of 24S-OHC were still significantly higher in MCI and AD patients compared to controls. The situation was the same with APOE and 27-OHC. It is evident that age is not a significant confounding variable in our study.

The levels of Tau, P-Tau ($p = 0.016$) and $A\beta_{1-42}$ in AD patients were significantly higher than in MCI patients. These three CSF markers were not significantly correlated to age, QAlb or CSF Albumin. Correction for age did not affect the observed differences between groups.

The highly significant correlation between levels of APOE and 24S-OHC found in patients with AD and patients with MCI is in accordance with the *in vitro* demonstration that 24S-OHC has a direct effect on APOE secretion from astrocytes (18). A similar strong correlation was not observed in healthy control subjects. A possible explanation could be that the coupling between 24S-OHC and APOE secretion is most important under conditions with increased levels of 24S-OHC in CSF. We and another group found that the levels of 24S-OHC in CSF are increased under conditions of neurodegeneration (100, 101), presumably reflecting populations of dying neuronal cells. Under these specific conditions the 24S-OHC-mediated increase in secretion of APOE may be more important than under normal conditions. APOE is a cholesterol-transporting lipoprotein, but we did not find a significant correlation between APOE and cholesterol in CSF from patients with neurodegeneration. However, a low but significant such correlation was observed in the control subjects.

Under *in vitro* conditions cholesterol increases cleavage of APP via the amyloidogenic pathway, whereas 24S-OHC has been reported to have an opposite effect (151, 152). If the neurodegenerative process results in increased levels of free

cholesterol, 24S-OHC may have a role both as a suppressor of amyloidogenesis and as a stimulator of APOE-mediated removal of cholesterol. It appears that our results are consistent with the possibility that 24S-OHC-mediated secretion of APOE results in an overcapacity for transport of sterols from CNS under conditions of neurodegeneration but not under normal conditions.

For reasons of comparison, we also included measurements of 27-OHC in our study. Most of this oxysterol present in CSF appears to originate from extracerebral sources (149). The lack of correlation between 27-OHC and APOE in the two groups of patients and in the controls is in accordance with this.

It is noteworthy that APOE was not correlated to 27-OHC, albumin or Qalb, markers of the BBB functionality. It may be concluded that the increased levels of APOE in the CSF of AD and MCI patients can not be simply addressed to a dysfunction of the BBB.

As described above the transcription of CYP46A1 has been found to be resistant to most regulatory factors tested, and availability of substrate cholesterol may be the most important factor under normal conditions (73). Oxidative stress was one of the few factors capable to up-regulate the gene at a transcriptional level (73). Since oxidative stress occurs in CNS of patients with AD (156), it is possible that this may contribute to increased formation of 24S-OHC and increased flux of APOE from the astrocytes.

Here we found that the CSF levels of APOE were significantly increased in AD and MCI patients. Previous measurements of APOE in CSF from patients with neurodegeneration have given contradictory results and increased (153-155), decreased (157-160) or unchanged levels have been reported (161). The different results observed by the different authors may be due to methodological differences and /or the characteristics of the control population used in each study.

In paper VI we showed that 24S-OHC is a promising marker for both early and advanced neurodegeneration (100, and *Paper VI).* The high correlation between 24S-OHC and APOE in CSF from such patients is consistent with the possibility that also APOE levels may be used diagnostically. At present, the level of T-Tau protein is generally used as a diagnostic marker for neurodegeneration (167) and it is demonstrated here that also the levels of T-Tau protein correlated to the APOE levels in our patients.

.

Paper VI

Can levels of 24S-OHC in CSF be used as a marker for neurodegeneration?

We tested the possibility that 24S-OHC in CSF may be used as a marker for neurodegeneration. The levels of 24S-OHC in CSF were found to be significantly positively correlated with t-tau as well as p-tau, considering all the patients together (Fig. 13 A and B). Similar correlations were found between 24S-OHC and t-tau and p-tau both in the AD and MCI cases. There was no significant correlation between CSF levels of 27-OHC and t-tau or p-tau, respectively.

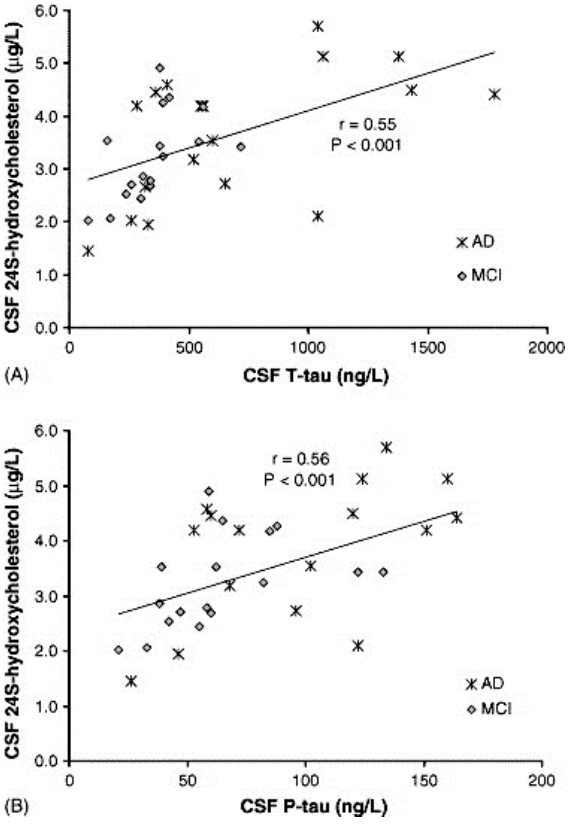


Figure 13. Correlation between CSF levels of 24S-OHC and total tau (t-tau) **(A)** and phospho-tau (p-tau) **(B)** protein in patients with mild cognitive impairment (MCI) and Alzheimer's disease (AD).

Based on the measurements in the controls, a 95% confidence interval was defined for the CSF 24S-OHC. The highest cut-off value, corresponding to the 95th percentile of the 24S-OHC distribution, was calculated to be >3.0 µg/L to distinguish normal from pathological values. The cut-off values for the CSF markers were set according to what has been published in the literature (64, 65); t-tau protein (>300 ng/L in the group 21–50 years of age, >450 ng/L in the group 51–70 years of age, and >500 ng/L in the groups 71–93 years of age), p-tau (>65 ng/L) and $A\beta_{42}$ (<500 ng/L). Individual values for each patient were compared with the cut-off levels.

In the AD patients, the percentage of subjects with increased levels of 24S-OHC (67%), t-tau (61%), p-tau (69%) and decreased $A\beta_{42}$ (56%) were similar. In the case of MCI, 50% of the patients had increased CSF levels of 24S-OHC, whereas the 18% and 29% of this population had increased levels of t-tau and p-tau, respectively. Decreased levels of $A\beta_{42}$ were found in one of the MCI patients (Figure 14).

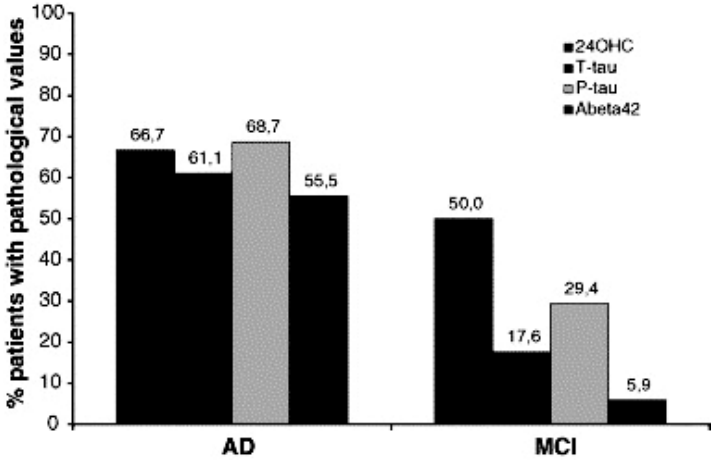


Figure 14. Proportion of the patients in the different diagnostic groups with pathological levels of the markers in CSF. The proportion was calculated as the number of subjects with increased or decreased values divided by the number of subjects in each group and expressed in percentage. For 24S-hydroxycholesterol (24S-OHC) the upper cut-off value was calculated as the 95th percentile value in the control group (3 μg/L). The cut-off values of t-tau, p-tau and Aβ42 were taken from the literature. t-tau > 300 ng/L (21–50 years) or 450 (51–70 years) or 500 ng/L (71–93 years); p-tau > 65 ng/L and Aβ42 < 500 ng/L.

In previous work (93) it was found that neuronal damage causes increased CSF levels of 24S-OHC, and that a dysfunction of the blood–brain and blood–CSF barriers results in increased levels of 27-OHC. In this study, significantly increased levels of both 24- and 27-OHC were found in patients diagnosed with AD and MCI (the two group of patients studied), compared to controls.

Furthermore, in both groups we found that in a high fraction of patients the levels of 24S-OHC were increased over the set cut-off. The high fraction of MCI patients with increased CSF 24S-OHC (50%), as compared with the smaller number of the same patients with a significant alteration of the levels of the other markers, is consistent with the possibility that 24S-OHC might be used as a new biomarker for ongoing neurodegeneration in the evaluation of patients with MCI. Whether or not elevated values can predict development into AD has to be evaluated in longitudinal studies where the conversion from MCI to AD must be assessed. The MCI cohort used in the present study was diagnosed according to the latest criteria suggested by Winblad et al. (164). It should be emphasised that the MCI group is heterogeneous and this heterogeneity might explain the non-Alzheimer distribution of APOE4.

The level of CSF t-tau is regarded to reflect the degree of neuronal degeneration and damage. In addition to indicating neuronal damage, CSF p-tau is believed to reflect phosphorylation of tau and thus formation of neurofibrillary tangles (167).

As expected, the "neuronal" 24S-OHC, a supposed marker of ongoing neuronal destruction was found to be significantly positively correlated with both t-tau and p-tau both in patients with AD and with MCI. Interestingly, there was no such correlation between the CSF level of 27-OHC and the other markers, in accordance with the contention that most of the 27-OHC present in CSF originates from the circulation rather than from the brain (149, 162).

However, the levels of 27-OHC were found to be significantly increased in both MCI and AD patients as compared to the controls, in according with a previous report (100). The enzyme responsible for the synthesis of the 27-OHC, the CYP27A1 has been found to be expressed in the brain, but 27-OHC was found to be 10% of the levels of 24S-OHC (166). The increased levels of 27-OHC may be due to dysfunction in the blood–brain and blood–CSF barriers. It has been reported that patients with AD may have a BBB or blood–CSF barrier dysfunction (163). Why this was also present in MCI subjects is not clear. It might reflect that MCI subjects may have brain disorders such as pre-AD and or cerebro-vascular disorders. Another possible explanation is that an increased flux of 24S-OHC as a result of neurodegeneration may have an inhibitory effect on the metabolism of 27-OHC as suggested by the results present in *paper IV*.

The increase in levels of 24S-OHC in CSF of patient with neurodegeneration must be discussed in relation to the fact that the plasma levels of the same oxysterol are decreased in the same patients. More than 99% of the 24S-OHC fluxing from the brain to the circulation passes the blood brain barrier and less than 1% passes via CSF. Most of the 24S-OHC present in the CSF is bound to APOE. Dying neuronal cells release cholesterol that will be "cleaned up" by the glial cells. Because of CYP46A1 induced in the latter cells, there will probably be a local elevation in the concentration of 24S-OHC that may stimulate synthesis and release of APOE from the astrocytes. The most important elimination of APOE from the brain is likely to be through cerebrospinal fluid. For this reason, there may be a preference for elimination of 24S-OHC from dying neuronal cells through cerebrospinal fluid, and it is possible that the local increase of APOE may be a driving force for this. The correlation between APOE and 24S-OHC in CSF from patients with AD and MCI but not in CSF from controls *(paper V)* is in accordance with this.

In conclusion, the CSF levels of 24S-OHC in this population of subjects indicate that 24S-OHC might be a specific marker of MCI. The present pilot study on a small number of patients does not allow an accurate calculation of diagnostic sensitivity and specificity. The significance of 24S-OHC as a marker has to be further tested in longitudinal studies where the conversion to AD is assessed in the MCI group. The relation between pre-clinical AD and high levels of CSF 24S-OHC will be evaluated including calculations of sensitivity and specificity.

The putative diagnostic and prognostic value of CSF 24S-OHC must also be compared with the corresponding values of t-tau, p-tau and $A\beta_{42}$.

Concluding remarks

The present thesis focuses on the regulation of the neuronal-specific enzyme cholesterol 24S-hydroxylase, the enzyme responsible for conversion of cholesterol to 24S-hydroxycholesterol. The regulatory role of 24S-OHC in cholesterol homeostasis in the brain has also been studied.
A related aim was to investigate the possible use of 24S-OHC as a marker for neurodegeneration. The following questions were addressed in the thesis and the results and conclusions are summarized below.

Is there a regulation of CYP46A1 at the epigenetic level?

CYP46A1 has proved resistant to previous attempts to modulate its expression with treatments known to modulate various signalling pathways of importance for cholesterol balance. In the present work we tested the possibility that epigenetic factors are of importance for its expression. We could demonstrate both *in vivo* and *vitro* that the histone deacetylase inhibitors Valproate and Trichostatin A induce the expression of CYP46A1, essentially reprogramming non-neuronal cells to express CYP46A1 to the same level as that found in adult neurons. These findings suggest that it is possible to modulate CYP46A1 in the brain by pharmacological means.

Is the drug Voriconazole also an inhibitor of CYP46A1 in vivo? Does the use of this drug cause changes in brain cholesterol homeostasis?

The crystal structure of CYP46A1 was recently determined and it was shown that several compounds can inhibit CYP46A1 *in vitro*. One of the most potent inhibitors found was the antifungal drug Voriconazole, which has the reported side effects of visual disturbances. As this drug is known to be able to enter the brain and as CYP46A1 is expressed in the retina, we hypothesised that inhibition of CYP46A1 may have a role in the reported side effects. Treatment of mice with Voriconazole led to a decrease in the concentration of 24S-OHC in the brain and a reduction in the lathosterol:cholesterol ratio, an index of brain cholesterol synthesis. In this connection it is of interest that in preliminary work pathological electroretinograms have been recorded from mice with a knockout of CYP46A1.

Is there a dietary regulation of CYP46A1 by omega-3 fatty acids?

Polyunsaturated fatty acids are essential structural components of the central nervous system and are of importance for learning and memory. A high ratio between omega-3 and omega-6 fatty acids seems to be an advantage. Two diets simulating the recommendations of the American Heart Association to increase the intake of n-3 polyunsaturated fatty acids were tested on Golden Syrian hamsters. CYP46A1 mRNA levels were increased in the liver and brain. There was a trend to increased cholesterol turnover in the brain as indicated by the slightly increased levels of lathosterol and 24S-OHC but unchanged levels of cholesterol. Increased CYP46A1 mRNA level is in line with the results of many human studies indicating that fish consumption or intake of n-3 polyunsaturated fatty acids have beneficial effects on brain function.

What are the metabolic consequences of an upregulation of CYP46A1?

In order to test the hypothesis that increased formation of 24S-OHC is neuroprotective and learn more about the regulatory importance of this oxysterol, transgenic mice overexpressing human CYP46A1 were generated and characterized. CYP46A1 mRNA levels were detected in the following organs: brain, eye, ovary, testis, kidney, lung and liver. Significant levels of CYP46A1 protein were however found only in brain, testis and eye with more than 10-fold higher levels in the brain than in the other organs. The circulating levels of 24S-OHC were increased by a factor of 4-6 and the fecal excretion of this steroid in free form was increased more than 20-fold. In the brain of the overexpressing mice the total amount of CYP46A1 protein was increased 2-4 fold and the expressed human enzyme protein had the same cellular distribution as the endogenous enzyme. The level of 24S-OHC in the brain was about double that of the wildtype. Increased activity of CYP46A1 would be expected to consume cholesterol with subsequent increased synthesis. In accordance with this brain levels of lathosterol and other cholesterol precursors were significantly increased. Under *in vitro* conditions 24S-OHC is an efficient activator of LXR. The overexpression did not however cause significant upregulation of LXR target genes in brain or liver. Whether or not overexpression of CYP46A1 has a protective effect in the activity on neurodegeneration and amyloid accumulation in the brain (*in vivo*) remains to be investigated.

An interaction between 24S-OHC and APOE has been demonstrated in vitro. Is there a correlation between APOE and 24OHC in cerebrospinal fluid?

One of the earliest observations to link cholesterol homeostasis with neurodegenerative diseases was the recognition that the ε4 isotype of APOE is an important risk factor for late-onset Alzheimer's disease. Recent in vitro work suggests that 24S-OHC may stimulate APOE transcription, protein synthesis and secretion. In light of this data, we hypothesised that there could be a correlation between the concentration of APOE and 24S-OHC in cerebrospinal fluid. In support of this hypothesis we found a significant such correlation in patients with AD and MCI, but not in the control group. The results are consistent with a close coupling between the formation and release of 24S-OHC and APOE secretion under conditions of ongoing neurodegeneration.

Can levels of 24S-OHC in CSF be used as a marker for neurodegeneration?

Given the above correlations, we investigated if the 24S-OHC in the cerebrospinal fluid is an early marker for AD. In support of our hypothesis, we could show that the cerebrospinal fluid content of 24S-OHC is at least as sensitive a marker for neurodegeneration in patients with AD as the accepted biomarkers (167) (i.e. tau,phospho-tau and β-amyloid). The significance of 24S-OHC as an early marker for neurodegeneration is now further tested in longitudinal studies.

ACKNOWLEDGEMENTS

From my own experience I know that these next couple of pages are the most read in the whole thesis. The moment of truth, for you as a reader to find out whether or not you have affected the life of this PhD candidate in someway.
Six years has almost passed since I came to the department of Clinical Chemistry to do my masters thesis. Little did I know about what being a PhD-candidate really ment; a lot of blood, sweat and occasionally some tears. As Piaf sang "Non, Je Ne Regrette Rien"!
These past few years have resulted in self development and personal growth for me, not to mention experiences that I will benefit from my whole life.
This thesis is the result of support, hard work and endless efforts made by a large number of individuals whom without, this would not have been possible. I would like to take this chance to express my deepest gratitude to everyone that has made a contribution.

First and foremost my supervisor **Professor Ingemar Björkhem**, for giving me this opportunity to be part of your research group. I am grateful to have learnt the true meaning of research from you. Your passion and knowledge in science, especially in the field of biochemistry is admirable. Thank you for everything!

My co-supervisor **Dr. Steve Meaney**, I am thankful for all the support and encouragement during these years, particularly in times of need. Thank you for sharing your ideas and theories with me. I have to admit, sometimes I don't understand them all but those I do grasp, I do carry out!

My other co-supervisor **Dr. Valerio Leoni**, thank you for introducing me to the field of oxysterol when I was a MSc. student. Thank you for all the discussions about everything from life to science. Thank you for being there for me at all times.

My last co- supervisor **Dr. Ulf Diczfalusy**, for always having time and responding all my questions about structural chemistry and references. Than you for all your effort with the thesis.

Head of the Department of Clinical Chemistry, **Dr. Paolo Parini**, for sharing your knowledge. Thank you for all the constructive discussions regarding methods, scientific approaches and medical statistics.

Maria Olin, thank you for being my side-kick during this time. I really would have been lost without you. Thank you for all your hard work and effort in connection with the different projects. Also thank you for always laughing with me!

Professor Stefan Alexsson for always having time for discussions regarding science, meetings, food, golf, tennis and carrier tactic

My colleague and good friend **Dr. Maura Heverin**, thank you for spreading positive energy in the lab with your PCC (Presence, Cakes and Candies). You always put a smile on my face!

Dr. Ann Båvner and **Dr.Dr. Magnus Hansson** thank you for all the laughs and good times. Not to mention the scientific expertise. Thanks guys!

Ulla Andersson, Anita Lövgren-Sandblom thank you for all the technical support in connection with the GCMS. Also for a memorable ski-weekend in Valbo! It was good fun!

Inger Moberg, thank you for taking such good care of me during the first weeks in the laboratory.

My friends and colleagues in the lab; **Dr.Lisa-Mari Nilsson, Vera Tillander, Kristina Kannisto, Hanna Nylen, Dr. Hanna Petterson, Dr. Matteo Pedrelli, Dr. Maura Heverin** (again), **Dr.Xiaoli Hu, Dr. Camilla Pramfalk , Grazia Stomeo** and **Dr. Johan Sladeen** for all the good times and laughter between everyday work. The doktorand room is so empty without many of you ☹

Lillian Larsson, for always laughing at my rather silly jokes and comments! You are wonderful!

Jenny Bernström, you have been so good to me during these years, always helping me with different administrative and non administrative tasks. You are a star!

Former members of the department of Clinical Chemistry; **Dr. Charlotte Murphy, Dr. O'Byrne, Dr. Maria Watter, Dr. Padideh Davoudpoor, Dr. Zhao-Yan Jiang, Dr. Katharina Slätis** the lab is not the same without you guys either!

Dr. S-J Reilley for all the memorable and crazy times spent together, you are truly a good friend no matter the occasion. Thank you for making me feel good, the times I was down.

My students **Riona O'Driscoll** and **Rima Nayef**, thank you for teaching me a lot about my self and not to mention all the help regarding the two first papers.

All my **colleagues in the routine**, thank you for teaching me the importance of organized work.

My furry friends (not the Persians ;) and all the **people in AKM 3, 5 and 6. Prof. Mustapha Hassan and his staff**, for all the hard work at these departments. Research would not have been possible otherwise.

Professor Björn Rozell thank you for sharing your knowledge and always answering my questions. I and rest of KI will miss you. Good Luck in Copenhagen!

All my collaborators specially **Prof. Pikuleva, Dr. Miia Kivipelto** and **Prof. Lars-Olof Wahlund** and their group members for a fruitful collaboration.

My Irish friends particularly "The spuds and Sill" drama group; **Oliver, Padraig "Mr. D'arcy", Teresa, Marie, Mary, Eddie, Karl, Jack, Miriam, Sarah and Emma** for an unforgettable performance. I don't think I have had that much fun in a long time as I did during rehearsal of the play.

A special thanks to the girls in **Karolinska dance-group**. Practise makes perfect!

My non Irish friends (also referred to as Arash's friends); **Javeria & Hussain , Roya & Axel, Giza & Alex, Joacim and Malin** thank you so much for all the good times

with good company and good food! Thank you for endless laughing, endless political discussions and for understanding and accepting may rage against “Lapp-lisor and P-nissar”.

The Yousefis, thank you for all the good times spent together. You always make me feel as a member of your family. Mobasher and Gita, thanks for all the crazy dancing in random places. You are awesome!

Jaleh & Saad, Natalie and Martin I am a lucky girl for having you guys. I will always remember the good times in Södertälje and fun times spent in Dubai. Thanks for your hospitality. I love you guys!

Hoda & Kennet and Shirin for all your love and support. All the laughs and tears ☺.You are the best!

The Farhadi family, for all the nice time spent together in Tehran.

The Mokhtari family, for all the kindness and love. Thank you for all the good times spent together and hopefully many more to come.

From the bottom of my heart I would like to thank; **Mahyar, Nazanin**, and my beautiful niece **Nicole**. Thank you for never make me feel far away from home with your phone-calls and text messages although they sometimes come in the middle of the night for some reason !

My baby brother **Maziar**, for all the good times back home (Malmö) and always being there for me with love and support.

My **aunt Massi** and **uncle Ali,** my cousins **Ashkan, Affe, Mansour** and my **grandmother**. Dear sweet **Hanna, Asal +1 baby girl**. Thank you for everything.

Cousin **Hamed** for all the interesting conversations over the phone and in Iran. It’s soon your turn. Good luck with the PhD studies.

My parents **Habib & Shahla**, for all the encouragements, support and unconditional love. Words can’t describe how much I love you both. I am so proud of what you have overcome and accomplished together in these past 35 years. I know that it has not been easy. If I can accomplish half of what you have in life, I am satisfied. This work is dedicated to both of you with all my love.

Last but not least, my beloved **Arash**. You went from being one of my good friends to being the ONE and the best thing that happened to me, even better than chocolate ;) Thank you for loving and understanding me the way you do.

A special thanks to the national research network **Swedish Brain Power** for funding this research. Thank you for connecting researchers in the field of neurodegeneration.

This research was also funded by **Vetenskapsrådet** and **Söderbergs stiftelse.**

REFERENCES

1. Vance DE, Van den Bosch H. Cholesterol in the year 2000. Biochim Biophys Acta 2000 Dec 15;1529(1-3):1-8.

2. Bjorkhem I, Meaney S. Brain cholesterol: long secret life behind a barrier. Arterioscler Thromb Vasc Biol 2004 May;24(5):806-15.

3. Vaillant C, Monard D. SHH pathway and cerebellar development. Cerebellum 2009 Sep;8(3):291-301.

4. Kushwaha RS, McGill HC, Jr. Mechanisms controlling lipemic responses to dietary lipids. World Rev Nutr Diet 1997;80:82-125.

5. Rodwell VW, Nordstrom JL, Mitschelen JJ. Regulation of HMG-CoA reductase. Adv Lipid Res 1976;14:1-74.

6. Engelking LJ, Liang G, Hammer RE, Takaishi K, Kuriyama H, Evers BM, et al. Schoenheimer effect explained--feedback regulation of cholesterol synthesis in mice mediated by Insig proteins. J Clin Invest 2005 Sep;115(9):2489-98.

7. Gill S, Chow R, Brown AJ. Sterol regulators of cholesterol homeostasis and beyond: the oxysterol hypothesis revisited and revised. Prog Lipid Res 2008 Nov;47(6):391-404.

8. Istvan ES, Palnitkar M, Buchanan SK, Deisenhofer J. Crystal structure of the catalytic portion of human HMG-CoA reductase: insights into regulation of activity and catalysis. EMBO J 2000 Mar 1;19(5):819-30.

9. Horton JD, Goldstein JL, Brown MS. SREBPs: activators of the complete program of cholesterol and fatty acid synthesis in the liver. J Clin Invest2002 May;109(9):1125-31.

10. Hardie DG, Scott JW, Pan DA, Hudson ER. Management of cellular energy by the AMP-activated protein kinase system. FEBS Lett 2003 Jul 3;546(1):113-20.

11. Jo Y, Debose-Boyd RA. Control of cholesterol synthesis through regulated ER-associated degradation of HMG CoA reductase. Crit Rev Biochem Mol Biol Jun;45(3):185-98.

12. Bellosta S, Ferri N, Bernini F, Paoletti R, Corsini A. Non-lipid-related effects of statins. Ann Med 2000 Apr;32(3):164-76.

13. Russell DW, Setchell KD. Bile acid biosynthesis. Biochemistry 1992 May 26;31(20):4737-49.

14. Baldan A, Bojanic DD, Edwards PA. The ABCs of sterol transport. J Lipid Res 2009 Apr;50 Suppl:S80-5.

15. Edmond J, Korsak RA, Morrow JW, Torok-Both G, Catlin DH. Dietary cholesterol and the origin of cholesterol in the brain of developing rats. J Nutr 1991 Sep;121(9):1323-30.
16. Dietschy JM, Turley SD. Thematic review series: brain Lipids. Cholesterol metabolism in the central nervous system during early development and in the mature animal. J Lipid Res 2004 Aug;45(8):1375-97.
17. Ikonen E. Cellular cholesterol trafficking and compartmentalization. Nat Rev Mol Cell Biol 2008 Feb;9(2):125-38.
18. Abildayeva K, Jansen PJ, Hirsch-Reinshagen V, Bloks VW, Bakker AH, Ramaekers FC, et al. 24(S)-hydroxycholesterol participates in a liver X receptor-controlled pathway in astrocytes that regulates apolipoprotein E-mediated cholesterol efflux. J Biol Chem 2006 May 5;281(18):12799-808.
19. Meaney S, Bodin K, Diczfalusy U, Bjorkhem I. On the rate of translocation in vitro and kinetics *in vivo* of the major oxysterols in human circulation: critical importance of the position of the oxygen function. J Lipid Res 2002 Dec;43(12):2130-5.
20. Bjorkhem I, Lutjohann D, Breuer O, Sakinis A, Wennmalm A. Importance of a novel oxidative mechanism for elimination of brain cholesterol. Turnover of cholesterol and 24(S)-hydroxycholesterol in rat brain as measured with 18O2 techniques *in vivo* and in vitro. J Biol Chem 1997 Nov 28;272(48):30178-84.
21. Lund EG, Xie C, Kotti T, Turley SD, Dietschy JM, Russell DW. Knockout of the cholesterol 24-hydroxylase gene in mice reveals a brain-specific mechanism of cholesterol turnover. J Biol Chem 2003 Jun 20;278(25):22980-8.
22. Lund EG, Menke JG, Sparrow CP. Liver X receptor agonists as potential therapeutic agents for dyslipidemia and atherosclerosis. Arterioscler Thromb Vasc Biol 2003 Jul 1;23(7):1169-77.
23. Whitney KD, Watson MA, Collins JL, Benson WG, Stone TM, Numerick MJ, et al. Regulation of cholesterol homeostasis by the liver X receptors in the central nervous system. Mol Endocrinol 2002 Jun;16(6):1378-85.
24. Pfrieger, F.W., Cholesterol homeostasis and function in neurons of the central nervous system. Cell Mol Life Sci, 2003. 60(6): p. 1158-71.
25. Ladu MJ, Reardon C, Van Eldik L, Fagan AM, Bu G, Holtzman D, et al. Lipoproteins in the central nervous system. Ann N Y Acad Sci 2000 Apr;903:167-75.

26. Martins IJ, Berger T, Sharman MJ, Verdile G, Fuller SJ, Martins RN. Cholesterol metabolism and transport in the pathogenesis of Alzheimer's disease. J Neurochem 2009 Dec;111 (6):1275-308.
27. Bassett CN, Montine KS, Neely MD, Swift LL, Montine TJ. Cerebrospinal fluid lipoproteins in Alzheimer's disease. Microsc Res Tech 2000 Aug 15;50(4):282-6.
28. Elshourbagy NA, Liao WS, Mahley RW, Taylor JM. Apolipoprotein E mRNA is abundant in the brain and adrenals, as well as in the liver, and is present in other peripheral tissues of rats and marmosets. Proc Natl Acad Sci U S A 1985 Jan;82(1):203-7.
29. Kim J, Basak JM, Holtzman DM. The role of apolipoprotein E in Alzheimer's disease. Neuron 2009 Aug 13;63(3):287-303.
30. Grehan S, Tse E, Taylor JM. Two distal downstream enhancers direct expression of the human apolipoprotein E gene to astrocytes in the brain. J Neurosci 2001 Feb 1;21(3):812-22.
31. Pitas RE, Boyles JK, Lee SH, Hui D, Weisgraber KH. Lipoproteins and their receptors in the central nervous system. Characterization of the lipoproteins in cerebrospinal fluid and identification of apolipoprotein B,E(LDL) receptors in the brain. J Biol Chem 1987 Oct 15;262(29):14352-60.
32. Strittmatter WJ, Bova Hill C. Molecular biology of apolipoprotein E. Curr Opin Lipidol 2002 Apr;13(2):119-23.
33. Mahley RW. Apolipoprotein E: cholesterol transport protein with expanding role in cell biology. Science1988 Apr 29;240(4852):622-30.
34. Cedazo-Minguez A. Apolipoprotein E and Alzheimer's disease: molecular mechanisms and therapeutic opportunities. J Cell Mol Med 2007 Nov Dec;11(6):1227-38.
35. Ashford JW. APOE genotype effects on Alzheimer's disease onset and epidemiology. J Mol Neurosci 2004;23(3):157-65.
36. Namba Y, Tomonaga M, Kawasaki H, Otomo E, Ikeda K. Apolipoprotein E immunoreactivity in cerebral amyloid deposits and neurofibrillary tangles in Alzheimer's disease and kuru plaque amyloid in Creutzfeldt-Jakob disease. Brain Res 1991 Feb 8;541(1):163-6.
37. Poirier J, Davignon J, Bouthillier D, Kogan S, Bertrand P, Gauthier S. Apolipoprotein E polymorphism and Alzheimer's disease. Lancet 1993 Sep 18;342(8873):697-9

38. Corder EH, Saunders AM, Risch NJ, Strittmatter WJ, Schmechel DE, Gaskell PC, Jr., et al. Protective effect of apolipoprotein E type 2 allele for late onset Alzheimer disease. Nat Genet1 994 Jun;7(2):180-4.
39. Schmechel DE, Saunders AM, Strittmatter WJ, Crain BJ, Hulette CM, Joo SH, et al. Increased amyloid beta-peptide deposition in cerebral cortex as a consequence of apolipoprotein E genotype in late-onset Alzheimer disease. Proc Natl Acad Sci U S A 1993 Oct 15;90(20):9649-53.
40. Wisniewski T, Castano EM, Golabek A, Vogel T, Frangione B. Acceleration of Alzheimer's fibril formation by apolipoprotein E in vitro. Am J Pathol 1994 Nov;145(5):1030-5.
41. Bogdanovic N, Corder E, Lannfelt L, Winblad B. APOE polymorphism and clinical duration determine regional neuropathology in Swedish APP(670, 671) mutation carriers: implications for late-onset Alzheimer's disease. J Cell Mol Med 2002 Apr-Jun;6(2):199-214.
42. Strittmatter WJ, Weisgraber KH, Goedert M, Saunders AM, Huang D, Corder EH, et al. Hypothesis: microtubule instability and paired helical filament formation in the Alzheimer disease brain are related to apolipoprotein E genotype. Exp Neurol 1994 Feb;125(2):163-71; discussion 72-4.
43. DeMattos RB, Thorngate FE, Williams DL. A test of the cytosolic apolipoprotein E hypothesis fails to detect the escape of apolipoprotein E from the endocytic pathway into the cytosol and shows that direct expression of apolipoprotein E in the cytosol is cytotoxic. J Neurosci 1999 Apr 1;19(7):2464-73
44. Bellosta S, Nathan BP, Orth M, Dong LM, Mahley RW, Pitas RE. Stable expression and secretion of apolipoproteins E3 and E4 in mouse neuroblastoma cells produces differential effects on neurite outgrowth. J Biol Chem1 995 Nov 10;270(45):27063-71.
45. Jansen PJ, Lutjohann D, Thelen KM, von Bergmann K, van Leuven F, Ramaekers FC, et al. Absence of ApoE upregulates murine brain ApoD and ABCA1 levels, but does not affect brain sterol levels, while human ApoE3 and human ApoE4 upregulate brain cholesterol precursor levels. J Alzheimers Dis 2009 Oct;18(2):319-29.
46. Calero M, Rostagno A, Matsubara E, Zlokovic B, Frangione B, Ghiso J. Apolipoprotein J (clusterin) and Alzheimer's disease. Microsc Res Tech 2000 Aug 15;50(4):305-15.

47. De Silva HV, Harmony JA, Stuart WD, Gil CM, Robbins J. Apolipoprotein J: structure and tissue distribution. Biochemistry 1990 Jun 5;29(22):5380-9.
48. De Silva HV, Stuart WD, Park YB, Mao SJ, Gil CM, Wetterau JR, et al. Purification and characterization of apolipoprotein J. J Biol Chem 1990 Aug 25;265(24):14292-7.
49. Collard MW, Griswold MD. Biosynthesis and molecular cloning of sulfated glycoprotein 2 secreted by rat Sertoli cells. Biochemistry 1987 Jun 16;26(12):3297-303.
50. Danik M, Chabot JG, Hassan-Gonzalez D, Suh M, Quirion R. Localization of sulfated glycoprotein-2/clusterin mRNA in the rat brain by in situ hybridization. J Comp Neurol 1993 Aug 8;334(2):209-27.
51. Wu E, Brosnan CF, Raine CS. SP-40,40 immunoreactivity in inflammatory CNS lesions displaying astrocyte/oligodendrocyte interactions. J Neuropathol Exp Neurol 1993 Mar;52(2):129-34.
52. Singhrao SK, Neal JW, Morgan BP, Gasque P. Increased complement biosynthesis by microglia and complement activation on neurons in Huntington's disease. Exp Neurol 1999 Oct;159(2):362-76.
53. Choi-Miura NH, Ihara Y, Fukuchi K, Takeda M, Nakano Y, Tobe T, et al. SP-40,40 is a constituent of Alzheimer's amyloid. Acta Neuropathol1992;83(3):260-4.
54. Grassilli E, Bettuzzi S, Troiano L, Ingletti MC, Monti D, Corti A, et al. SGP-2, apoptosis, and aging. Ann N Y Acad Sci1 992 Nov 21;663:471-4.
55. Duguid JR, Bohmont CW, Liu NG, Tourtellotte WW. Changes in brain gene expression shared by scrapie and Alzheimer disease. Proc Natl Acad Sci U S A 1989 Sep;86(18):7260-4.
56. Morgan TE, Laping NJ, Rozovsky I, Oda T, Hogan TH, Finch CE, et al. Clusterin expression by astrocytes is influenced by transforming growth factor beta 1 and heterotypic cell interactions. J Neuroimmunol 1995 Apr;58(1):101-10.
57. McConathy WJ, Alaupovic P. Isolation and partial characterization of apolipoprotein D: a new protein moiety of the human plasma lipoprotein system. FEBS Lett 1973; 37:178-82.
58. Fan QW, Yu W, Gong JS, Zou K, Sawamura N, Senda T, et al. Cholesterol-dependent modulation of dendrite outgrowth and microtubule stability in cultured neurons. J Neurochem 2002 Jan;80(1):178-90.
59. Muffat J, Walker DW. Apolipoprotein D: an overview of its role in aging and age-related diseases. Cell Cycle Jan 15;9(2):269-73.

60. Muffat J, Walker DW, Benzer S. Human ApoD, an apolipoprotein up-regulated in neurodegenerative diseases, extends lifespan and increases stress resistance in Drosophila. Proc Natl Acad Sci U S A 2008 May 13;105(19):7088-93.
61. Björkhem I, Lutjohann D, Diczfalusy U, Stahle L, Ahlborg G, Wahren J. Cholesterol homeostasis in human brain: turnover of 24S-hydroxycholesterol and evidence for a cerebral origin of most of this oxysterol in the circulation. J Lipid Res. 1998;39:1594–1600
62. Björkhem , I. Are side-chain oxidized oxysterols regulators also in vivo? J Lipid Res, 2009.(4) p. 213-218.
63. Bjorkhem, I. and U. Diczfalusy, Oxysterols: friends, foes, or just fellow passengers? Arterioscler Thromb Vasc Biol, 2002. 22(5): p. 734-42.
64. Heverin M, Meaney S, Lutjohann D, Diczfalusy U, Wahren J, Bjorkhem I. Crossing the barrier: net flux of 27-hydroxycholesterol into the human brain. J Lipid Res 2005 May;46(5):1047-52.
65. Leoni V., On the possible use of oxysterols for the diagnosis and evaluation of patients with neurological and neurodegenerative diseases. Doctoral thesis, Stockholm 2005.
66. Leoni, V., et al., Side-chain oxidised oxysterols in cerebrospinal fluid and integrity of the blood–brain barrier, J. Lipid Res. 2003,.44, pp. 793–799.
67. Bjorkhem I, Araya Z, Rudling M, Angelin B, Einarsson C, Wikvall K. Differences in the regulation of the classical and the alternative pathway for bile acid synthesis in human liver. No coordinate regulation of CYP7A1 and CYP27A1. J Biol Chem 2002 Jul 26;277(30):26804-7.
68. Sawada N, Sakaki T, Kitanaka S, Kato S, Inouye K. Structure-function analysis of CYP27B1 and CYP27A1. Studies on mutants from patients with vitamin D-dependent rickets type I (VDDR-I) and cerebrotendinous xanthomatosis (CTX). Eur J Biochem 2001 Dec;268(24):6607-15.
69. Russell DW, Halford RW, Ramirez DM, Shah R, Kotti T. Cholesterol 24-hydroxylase: an enzyme of cholesterol turnover in the brain. Annu Rev Biochem 2009;78:1017-40.
70. Lund EG, Guileyardo JM, Russell DW. cDNA cloning of cholesterol 24-hydroxylase, a mediator of cholesterol homeostasis in the brain. Proc Natl Acad Sci U S A1999 Jun 22;96(13):7238-43.

71. Mast N, Norcross R, Andersson U, Shou M, Nakayama K, Bjorkhem I, et al. Broad substrate specificity of human cytochrome P450 46A1 which initiates cholesterol degradation in the brain. Biochemistry 2003 Dec 9;42(48):14284-92.
72. Mast N, White MA, Bjorkhem I, Johnson EF, Stout CD, Pikuleva IA. Crystal structures of substrate-bound and substrate-free cytochrome P450 46A1, the principal cholesterol hydroxylase in the brain. Proc Natl Acad Sci U S A 2008 Jul 15;105(28):9546-51.
73. Ohyama Y, Meaney S, Heverin M, Ekstrom L, Brafman A, Shafir M, et al. Studies on the transcriptional regulation of cholesterol 24-hydroxylase (CYP46A1): marked insensitivity toward different regulatory axes. J Biol Chem 2006 Feb 17;281(7):3810-20.
74. Zarrinpar A, Bhattacharyya RP, Lim WA. The structure and function of proline recognition domains. Sci STKE2003 Apr 22; 2003(179):RE8.
75. White MA, Mast N, Bjorkhem I, Johnson EF, Stout CD, Pikuleva IA. Use of complementary cation and anion heavy-atom salt derivatives to solve the structure of cytochrome P450 46A1. Acta Crystallogr D Biol Crystallogr 2008 May;64(Pt 5):487-95.
76. Lee J, Kosaras B, Aleyasin H, Han JA, Park DS, Ratan RR, et al. Role of cyclooxygenase-2 induction by transcription factor Sp1 and Sp3 in neuronal oxidative and DNA damage response. FASEB J 2006 Nov;20(13):2375-7.
77. Ryu H, Lee J, Zaman K, Kubilis J, Ferrante RJ, Ross BD, et al. Sp1 and Sp3 are oxidative stress-inducible, antideath transcription factors in cortical neurons. J Neurosci 2003 May 1;23(9):3597-606.
78. Milagre I, Nunes MJ, Gama MJ, Silva RF, Pascussi JM, Lechner MC, et al. Transcriptional regulation of the human CYP46A1 brain-specific expression by Sp transcription factors. J Neurochem 2008 Jul;106(2):835-49.
79. Lutjohann D, Bjorkhem I, Locatelli S, Dame C, Schmolling J, von Bergmann K, et al. Cholesterol dynamics in the foetal and neonatal brain as reflected by circulatory levels of 24S-hydroxycholesterol. Acta Paediatr2001 Jun;90(6):652-7.
80. Kotti TJ, Ramirez DM, Pfeiffer BE, Huber KM, Russell DW. Brain cholesterol turnover required for geranylgeraniol production and learning in mice. Proc Natl Acad Sci U S A 2006 Mar 7;103(10):3869-74.
81. Sasaki K, Yamagata T, Mitani K. Histone deacetylase inhibitors trichostatin A and valproic acid circumvent apoptosis in human leukemic cells expressing the RUNX1 chimera. Cancer Sci 2008 Feb;99(2):414-22.

82. Minucci S, Pelicci PG. Histone deacetylase inhibitors and the promise of epigenetic (and more) treatments for cancer. Nat Rev Cancer 2006 Jan;6(1):38-51.
83. Selkoe DJ. Alzheimer's disease: genes, proteins, and therapy. Physiol Rev 2001 Apr;81(2):741-66.
84. Puglielli L, Tanzi RE, Kovacs DM. Alzheimer's disease: the cholesterol connection. Nat Neurosci 2003 Apr;6(4):345-51.
85. Sparks DL, Scheff SW, Hunsaker JC, 3rd, Liu H, Landers T, Gross DR. Induction of Alzheimer-like beta-amyloid immunoreactivity in the brains of rabbits with dietary cholesterol. Exp Neurol 1994 Mar;126(1):88-94.
86. Gotz J, Ittner LM, Schonrock N, Cappai R. An update on the toxicity of Abeta in Alzheimer's disease. Neuropsychiatr Dis Treat 2008 Dec;4(6):1033-42.
87. Refolo LM, Malester B, LaFrancois J, Bryant-Thomas T, Wang R, Tint GS, et al. Hypercholesterolemia accelerates the Alzheimer's amyloid pathology in a transgenic mouse model. Neurobiol Dis 2000 Aug;7(4):321-31.
88. Papassotiropoulos A, Streffer JR, Tsolaki M, Schmid S, Thal D, Nicosia F, et al. Increased brain beta-amyloid load, phosphorylated tau, and risk of Alzheimer disease associated with an intronic CYP46 polymorphism. Arch Neurol 2003 Jan;60(1):29-35.
89. Borroni B, Archetti S, Agosti C, Akkawi N, Brambilla C, Caimi L, et al. Intronic CYP46 polymorphism along with ApoE genotype in sporadic Alzheimer Disease: from risk factors to disease modulators. Neurobiol Aging 2004 Jul;25(6):747-51.
90. Kolsch H, Lutjohann D, Jessen F, Popp J, Hentschel F, Kelemen P, et al. CYP46A1 variants influence Alzheimer's disease risk and brain cholesterol metabolism. Eur Psychiatry 2009 Apr;24(3):183-90.
91. Garcia AN, Muniz MT, Souza e Silva HR, da Silva HA, Athayde-Junior L. Cyp46 polymorphisms in Alzheimer's disease: a review. J Mol Neurosci2009 Nov;39(3):342-5.
92. Famer, D., et al., Regulation of alpha- and beta-secretase activity by oxysterols: cerebrosterol stimulates processing of APP via the alpha-secretase pathway. Biochem Biophys Res Commun, 2007. 359(1): p. 46-50.
93. Leoni, V., Oxysterols as markers of neurological disease--a review. Scand J Clin Lab Invest, 2009. 69(1): p. 22-5.
94. Bretillon L, Siden A, Wahlund LO, Lutjohann D, Minthon L, Crisby M, et al. Plasma levels of 24S-hydroxycholesterol in patients with neurological diseases. Neurosci Lett 2000 Oct 27;293(2):87-90.

95. Papassotiropoulos A, Lutjohann D, Bagli M, Locatelli S, Jessen F, Buschfort R, et al. 24S-hydroxycholesterol in cerebrospinal fluid is elevated in early stages of dementia. J Psychiatr Res 2002 Jan-Feb;36(1):27-32.
96. Papassotiropoulos A, Lutjohann D, Bagli M, Locatelli S, Jessen F, Rao ML, et al. Plasma 24S-hydroxycholesterol: a peripheral indicator of neuronal degeneration and potential state marker for Alzheimer's disease. Neuroreport 2000 Jun 26;11(9):1959-62.
97. Lutjohann D, Papassotiropoulos A, Bjorkhem I, Locatelli S, Bagli M, Oehring RD, et al. Plasma 24S-hydroxycholesterol (cerebrosterol) is increased in Alzheimer and vascular demented patients. J Lipid Res 2000 Feb;41(2):195-8.
98. Bogdanovic N, Bretillon L, Lund EG, Diczfalusy U, Lannfelt L, Winblad B, et al. On the turnover of brain cholesterol in patients with Alzheimer's disease. Abnormal induction of the cholesterol-catabolic enzyme CYP46 in glial cells. Neurosci Lett 2001 Nov 13;314(1-2):45-8.
99. Brown J, 3rd, Theisler C, Silberman S, Magnuson D, Gottardi-Littell N, Lee JM, et al. Differential expression of cholesterol hydroxylases in Alzheimer's disease. J Biol Chem 2004 Aug 13;279(33):34674-81.
100. Leoni V, Masterman T, Mousavi FS, Wretlind B, Wahlund LO, Diczfalusy U, et al. Diagnostic use of cerebral and extracerebral oxysterols. Clin Chem Lab Med2004 Feb;42(2):186-91.
101. Schonknecht P, Lutjohann D, Pantel J, Bardenheuer H, Hartmann T, von Bergmann K, et al. Cerebrospinal fluid 24S-hydroxycholesterol is increased in patients with Alzheimer's disease compared to healthy controls. Neurosci Lett2002 May 10;324(1):83-5.
102. Hooijmans CR, Kiliaan AJ. Fatty acids, lipid metabolism and Alzheimer pathology. Eur J Pharmacol2008 May 6;585(1):176-96.
103. Jicha GA, Markesbery WR. Omega-3 fatty acids: potential role in the management of early Alzheimer's disease. Clin Interv Aging;5:45-61.
104. de la Torre JC. Critically attained threshold of cerebral hypoperfusion: the CATCH hypothesis of Alzheimer's pathogenesis. Neurobiol Aging 2000 Mar-Apr;21(2):331-42.
105. Yehuda S, Rabinovitz S, Mostofsky DI. Essential fatty acids and the brain: from infancy to aging. Neurobiol Aging 2005 Dec;26 Suppl 1:98-102.
106. Auestad N. Infant nutrition--brain development--disease in later life. An introduction. Dev Neurosci 2000 Sep-Dec;22(5-6):472-3.

107. Folch J, Lees M, Sloane Stanley GH. A simple method for the isolation and purification of total lipides from animal tissues. J Biol Chem1957 May;226(1):497-509.
108. Folch J, Ascoli I, Lees M, Meath JA, Le BN. Preparation of lipide extracts from brain tissue. J Biol Chem1951 Aug;191(2):833-41.
109. Livak KJ, Schmittgen TD. Analysis of relative gene expression data using real-time quantitative PCR and the 2(-Delta Delta C(T)) Method. Methods2001 Dec;25(4):402-8.
110. Heverin M, Bogdanovic N, Lutjohann D, Bayer T, Pikuleva I, Bretillon L, et al. Changes in the levels of cerebral and extracerebral sterols in the brain of patients with Alzheimer's disease. J Lipid Res2004 Jan;45(1):186-93.
111. McKhann G, Drachman D, Folstein M, Katzman R, Price D, Stadlan EM. Clinical diagnosis of Alzheimer's disease: report of the NINCDS-ADRDA Work Group under the auspices of Department of Health and Human Services Task Force on Alzheimer's Disease. Neurology1984 Jul;34(7):939-44.
112. Winblad B, Palmer K, Kivipelto M, Jelic V, Fratiglioni L, Wahlund LO, et al. Mild cognitive impairment--beyond controversies, towards a consensus: report of the International Working Group on Mild Cognitive Impairment. J Intern Med2004 Sep;256(3):240-6.
113. Wahlund LO, Pihlstrand E, Jonhagen ME. Mild cognitive impairment: experience from a memory clinic. Acta Neurol Scand Suppl2003;179:21-4.
114. American Psychiatric Association, Diagnostic and Statistical Manual of Mental Disorders, 4th ed., American Psychiatric Association, Washington, DC, 1994.
115. Sanderson L, Taylor GW, Aboagye EO, Alao JP, Latigo JR, Coombes RC, et al. Plasma pharmacokinetics and metabolism of the histone deacetylase inhibitor trichostatin a after intraperitoneal administration to mice. Drug Metab Dispos2004 Oct;32(10):1132-8.
116. Li-Hawkins J, Lund EG, Bronson AD, Russell DW. Expression cloning of an oxysterol 7alpha-hydroxylase selective for 24-hydroxycholesterol. J Biol Chem2000 Jun 2;275(22):16543-9.
117. Ikeda H, Ueda M, Ikeda M, Kobayashi H, Honda Y. Oxysterol 7alpha-hydroxylase (CYP39A1) in the ciliary nonpigmented epithelium of bovine eye. Lab Invest2003 Mar;83(3):349-55.

118. Villagra A, Ulloa N, Zhang X, Yuan Z, Sotomayor E, Seto E. Histone deacetylase 3 down-regulates cholesterol synthesis through repression of lanosterol synthase gene expression. J Biol Chem 2007 Dec 7;282(49):35457-70.
119. Jeu L, Piacenti FJ, Lyakhovetskiy AG, Fung HB. Voriconazole. Clin Ther 2003 May;25(5):1321-81.
120. Lund , E. G. , C. Xie , T. Kotti , S. D. Turley , J. M. Dietschy , and D. W.Russell . Knockout of the cholesterol 24-hydroxylase gene in mice reveals a brain-specifi c mechanism of cholesterol turnover. J.Biol. Chem. 278 (2003) 22980 – 22988 .
121. Bretillon L, Diczfalusy U, Bjorkhem I, Maire MA, Martine L, Joffre C, et al. Cholesterol-24S-hydroxylase (CYP46A1) is specifically expressed in neurons of the neural retina. Curr Eye Res 2007 Apr;32(4):361-6.
122. Yoshida H, Ikeda I, Tomooka M, Mawatari M, Imaizumi K, Seto A, et al. Effect of dietary seal and fish oils on lipid metabolism in hamsters. J Nutr Sci Vitaminol (Tokyo)2001 Jun;47(3):242-7.
123. Spady DK, Horton JD, Cuthbert JA. Regulatory effects of n-3 polyunsaturated fatty acids on hepatic LDL uptake in the hamster and rat. J Lipid Res1995 May;36(5):1009-20.
124. .Surette ME, Whelan J, Lu GP, Broughton KS, Kinsella JE. Dependence on dietary cholesterol for n-3 polyunsaturated fatty acid-induced changes in plasma cholesterol in the Syrian hamster. J Lipid Res1992 Feb;33(2):263-71.
125. Lin MH, Lu SC, Huang PC, Liu YC, Liu SY. The amount of dietary cholesterol changes the mode of effects of (n-3) polyunsaturated fatty acid on lipoprotein cholesterol in hamsters. Ann Nutr Metab2004 Sep-Oct;48(5):321-8.
126. Bjorkhem I, Reihner E, Angelin B, Ewerth S, Akerlund JE, Einarsson K. On the possible use of the serum level of 7 alpha-hydroxycholesterol as a marker for increased activity of the cholesterol 7 alpha-hydroxylase in humans. J Lipid Res1987 Aug;28(8):889-94.
127. Nicolau G, Shefer S, Salen G, Mosbach EH. Determination of hepatic 3-hydroxy-3-methylglutaryl CoA reductase activity in man. J Lipid Res1974 Jan;15(1):94-8.
128. Reihner E, Angelin B, Rudling M, Ewerth S, Bjorkhem I, Einarsson K. Regulation of hepatic cholesterol metabolism in humans: stimulatory effects of cholestyramine on HMG-CoA reductase activity and low density lipoprotein receptor expression in gallstone patients. J Lipid Res1990 Dec;31(12):2219-26.

129. Oda H, Yamashita H, Kosahara K, Kuroki S, Nakayama F. Esterified and total 7 alpha-hydroxycholesterol in human serum as an indicator for hepatic bile acid synthesis. J Lipid Res1990 Dec;31(12):2209-18.
130. Russell DW. Fifty years of advances in bile acid synthesis and metabolism. J Lipid Res2009 Apr;50 Suppl:S120-5.
131. Horton JD, Cuthbert JA, Spady DK. Regulation of hepatic 7 alpha-hydroxylase expression and response to dietary cholesterol in the rat and hamster. J Biol Chem1995 Mar 10;270(10):5381-7.
132. De Fabiani E, Crestani M, Marrapodi M, Pinelli A, Chiang JY, Galli G. Regulation of the hamster cholesterol 7 alpha-hydroxylase gene (CYP7A): prevalence of negative over positive transcriptional control. Biochem Biophys Res Commun1996 Sep 24;226(3):663-71.
133. Gupta S, Pandak WM, Hylemon PB. LXR alpha is the dominant regulator of CYP7A1 transcription. Biochem Biophys Res Commun2002 Apr 26;293(1):338-43.
134. Chico Y, Fresnedo O, Lacort M, Ochoa B. Effect of estradiol and progesterone on cholesterol 7 alpha-hydroxylase activity in rats subjected to different feeding conditions. Steroids1994 Sep;59(9):528-35.
135. Stroeve JH, Brufau G, Stellaard F, Gonzalez FJ, Staels B, Kuipers F. Intestinal FXR-mediated FGF15 production contributes to diurnal control of hepatic bile acid synthesis in mice. Lab Invest Jun 7.
136. Noshiro M, Nishimoto M, Okuda K. Rat liver cholesterol 7 alpha-hydroxylase. Pretranslational regulation for circadian rhythm. J Biol Chem1990 Jun 15;265(17):10036-41.
137. Mayer D. The circadian rhythm of synthesis and catabolism of cholesterol. Arch Toxicol 1976 Dec 17;36(3-4):267-76.
138. Heverin M, Bogdanovic N, Lutjohann D, Bayer T, Pikuleva I, Bretillon L, et al. Changes in the levels of cerebral and extracerebral sterols in the brain of patients with Alzheimer's disease. J Lipid Res2004 Jan;45(1):186-93.
139. Pedersen JI, Oftebro H, Bjorkhem I. Reconstitution of C27-steroid 26-hydroxylase activity from bovine brain mitochondria. Biochem Int1989 Mar;18(3):615-22.
140. Dietschy JM. Central nervous system: cholesterol turnover, brain development and neurodegeneration. Biol Chem2009 Apr;390(4):287-93.
141. Kris-Etherton PM, Hecker KD, Binkoski AE. Polyunsaturated fatty acids and cardiovascular health. Nutr Rev 2004 Nov;62(11):414-26.

142. Ramirez DM, Andersson S, Russell DW. Neuronal expression and subcellular localization of cholesterol 24-hydroxylase in the mouse brain. J Comp Neurol 2008 Apr 10;507(5):1676-93.

143. Hudry E, Van Dam D, Kulik W, De Deyn PP, Stet FS, Ahouansou O, et al. Adeno-associated virus gene therapy with cholesterol 24-hydroxylase reduces the amyloid pathology before or after the onset of amyloid plaques in mouse models of Alzheimer's disease. Mol Ther Jan;18(1):44-53.

144. Morris MC, Evans DA, Bienias JL, Tangney CC, Bennett DA, Wilson RS, et al. Consumption of fish and n-3 fatty acids and risk of incident Alzheimer disease. Arch Neurol 2003 Jul;60(7):940-6.

145. Uppal H, Saini SP, Moschetta A, Mu Y, Zhou J, Gong H, et al. Activation of LXRs prevents bile acid toxicity and cholestasis in female mice. Hepatology 2007 Feb;45(2):422-32.

146. Bjorkhem I, Andersson U, Ellis E, Alvelius G, Ellegard L, Diczfalusy U, et al. From brain to bile. Evidence that conjugation and omega-hydroxylation are important for elimination of 24S-hydroxycholesterol (cerebrosterol) in humans. J Biol Chem 2001 Oct 5;276(40):37004-10.

147. Meaney S, Lutjohann D, Diczfalusy U, Bjorkhem I. Formation of oxysterols from different pools of cholesterol as studied by stable isotope technique: cerebral origin of most circulating 24S-hydroxycholesterol in rats, but not in mice. Biochim Biophys Acta 2000 Jul 19;1486(2-3):293-8.

148. Riddell DR, Zhou H, Comery TA, Kouranova E, Lo CF, Warwick HK, et al. The LXR agonist TO901317 selectively lowers hippocampal Abeta42 and improves memory in the Tg2576 mouse model of Alzheimer's disease. Mol Cell Neurosci 2007 Apr;34(4):621-8.

149. Leoni V, Masterman T, Patel P, Meaney S, Diczfalusy U, Bjorkhem I. Side chain oxidized oxysterols in cerebrospinal fluid and the integrity of blood-brain and blood-cerebrospinal fluid barriers. J Lipid Res 2003 Apr;44(4):793-9.

150. Heverin M, Meaney S, Brafman A, Shafir M, Olin M, Shafaati M, et al. Studies on the cholesterol-free mouse: strong activation of LXR-regulated hepatic genes when replacing cholesterol with desmosterol. Arterioscler Thromb Vasc Biol 2007 Oct;27(10):2191-7.

151. Brown, J., et al., Differential expression of cholesterol hydroxylases in Alzheimer's disease. J Biol Chem 2004; 279: 34674-34681.

152. Famer, D., et al., Regulation of alpha- and beta-secretase activity by oxysterols: cerebrosterol stimulates processing of APP via the alpha-secretase pathway. Biochem Biophys Res Commun, 2007. 359(1): p. 46-50.
153. Fukuyama, R., et al., Age-dependent decline in the apolipoprotein E level in cerebrospinal fluid from control subjects and its increase in cerebrospinal fluid from patients with Alzheimer's disease, Eur. Neurol. 43 (2000), pp. 161–169.
154. Lindh, M., et al., Cerebrospinal fluid apolipoprotein E (apoE) levels in Alzheimer's disease patients are increased at follow up and show a correlation with levels of Tau protein, Neurosci. Lett. 229 (1997), pp. 85–88.
155. Merched, A., et al., Cerebrospinal fluid apolipoprotein e level is increased in late-onset Alzheimer's disease, J. Neuro. Sci. 145 (1997), pp. 33–39.
156. Moreira, P.I., et al., Oxidative stress and neurodegeneration, Ann. NY Acad. Sci. 1043 (2005), pp. 545–552. (Review)
157. Blennow, K., et al., Cerebrospinal fluid apolipoprotein E is reduced in Alzheimer's disease, Neuroreport 5 (1994), pp. 2534–2536
158. Hesse, C., et al., Measurement of apolipoprotein E (apoE) in cerebrospinal fluid, Neurochem. Res. 25 (2000), pp. 511–517.
159. Landén, M., et al., Apolipoprotein E in cerebrospinal fluid from patients with Alzheimer's disease and other forms of dementia is reduced but without any correlation to the apoe4 isoform, Dementia 7 (1996), pp. 273–278.
160. Pirttilä, T., et al., Relationship between apolipoprotein E4 allele and CSF amyloid b-protein in Alzheimer's disease and controls, Neurosci. Res. Commun. 15 (1994), pp. 201–207
161. Hahne, S., et al., Levels of cerebrospinal fluid apolipoprotein E in patients with Alzheimer's disease and healthy controls, Neurosci. Lett. 224 (1997), pp. 99–102.
162. Bjorkhem, I., Crossing the barrier: oxysterols as cholesterol transporters and metabolic modulators in the brain. J Intern Med, 2006. 260(6): p. 493-508.
163. Blennow, K., et al., CSF markers for incipient Alzheimer's disease, Lancet Neurol. 2 (2003), pp. 605–613.
164. Winblad, D., et al., Mild cognitive impairment—beyond controversies, towards a consensus: report of the international Working Group on Mild Cognitive Impairment, J. Intern. Med. 2004, 256 pp. 240–246.
165. Blennow, K., et al., Blood brain barrier disturbance in patients with Alzheimer's disease is related to vascular factors, Acta Neurol. Scand. 81 (1990), pp. 323–326.

166.Heverin, M., et al., Changes in the levels of cerebral and extracerebral sterols in the brain of patients with Alzheimer's disease, J. Lipid Res. 2004; 45, pp. 186–193.
167.Trojanowski JQ, Vandeerstichele H, Korecka M, Clark CM, Aisen PS, Petersen RC, et al. Update on the biomarker core of the Alzheimer's Disease Neuroimaging Initiative subjects. Alzheimers Dement May;6(3):230-8.